ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section A PATHOLOGY



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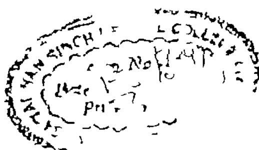
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THE ROLE OF THE PARATHYROIDS FOR THE ADAPTATION TO A LOW CALCIUM INTAKE

1 The Short Term Effect of Parathyroidectomy on the Adaptation to a Low Calcium Intake in Adult Rats with Special Reference to Plasma Calcium Bone Tissue and Adrenal Glands

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Larsson, S E. & Ahlgren, O The role of the parathyroids for the adaptation to a low calcium intake. 1 The short term effect of parathyroidectomy on the adaptation to a low calcium intake in adult rats with special reference to plasma calcium, bone tissue and adrenal glands. Acta path. microbiol. scand. Sect. A, 83 1–1 1975.

One-year-old selectively parathyroidectomized rats were found to have lost the ability to adapt themselves to a reduced calcium intake. Of importance in this respect was certainly the demonstrated inability to mobilize skeletal calcium to the blood. Thus, when the normal intake of calcium in the diet is reduced in the adult intact rat, skeletal calcium reserves must become mobilized with resulting osteoporosis. Histological and morphometric analyses of the adrenal glands showed no apparent effect of the parathyroidectomy upon the functional state of the adrenal cortex.

Key words Calcium deficiency calcium metabolism parathyroidectomy; osteoporosis; adrenal glands.

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Previous studies in our laboratory of the adaptation to a low calcium intake have demonstrated the occurrence of osteoporosis (*Larsson* 1969) and parathyroid hyperplasia (*Semastikoglou & Larsson* 1972) in adult rats during prolonged low calcium intake. Although a marked adaptation, viz. a distinctly increased efficiency of calcium absorption occurred there was a definite loss of bone tissue from the mature skeleton due to increased mobilization of bone calcium (*Larsson* 1969)

Vitamin D has been shown to be essential for the adaptation to a reduced calcium intake in young rats by increasing calcium absorption (*Nicolaysen et al* 1953) Although the occurrence of parathyroid hyperplasia indicates that the parathyroids are of importance for the mobilization of skeletal calcium the question whether endogenous parathyroid hormone has an influence on gastrointestinal absorption of calcium is not completely resolved (cf Ahlgren & Larsson 1975) The parathyroids have also been suggested to act as a major regulator of calcium absorption by regulating the synthesis of the metabolically active form of vitamin D 1,25-

dihydroxycholecalciferol, in response to changes in calcium intake and plasma calcium level (*Garabedian et al.* 1972).

The present investigation was performed in order to obtain information about the role played by the parathyroids during the adaptation to a low calcium intake in the adult individual. As in our previous studies, adult rats were used since most previous investigations of the adaptation of the calcium metabolism have been done on young rats. The present communication is part of a series of investigations of the effects of selective parathyroidectomy on calcium metabolism and the skeleton at normal and low intakes of calcium in the diet. In addition, the effect on the adrenal cortex was studied morphologically with regard to the possibility of an interrelationship between adrenal cortex and the parathyroids. In the present study the *short-term* effects on the plasma calcium level, the body weight, the adrenal cortex, the calcium accretion rate by bone and the bone mass are reported. A subsequent study of the same animals will deal with the effects on the calcium metabolism. Similar *long-term* studies will be reported elsewhere.

MATERIALS AND METHODS

Experimental Animals

A total number of 137 adult male rats were randomly divided into a short term series of 40 animals comprising 8 weeks and a long-term experimental series of 97 animals comprising 6 months. The latter series will be reported on in a subsequent communication. The animals were sterile bred rats of the Sprague-Dawley strain and were all stated to be 12 to 12½ months old at the start of the experiment. Their initial body weights were 515–645 grams. All animals had been bred on a standard laboratory ration described in detail (mixture 1) previously (*Eggum & Møllegaard Hansen* 1967). The mineral mixture amounted to 2.0 per cent and contained 83.00 per cent di-calciumphosphat. The content of vitamin D was 1,000 I.U. per 100 g diet**.

Housing

Without access to direct sunlight, all the animals were housed five to each cage at a temperature of 18–20° C. Macrolone cages with a heap of chips on the floor were used and the animals could move about freely.

Experimental Diets

The low calcium diet of General Biochemical Inc., Chagrin Falls, Ohio, was used as a basic ration. The composition and ingredients of this diet (GBI Bulletin D-15) has been described in detail elsewhere (*Larsson* 1969). Chemical analyses showed that the diet contained 0.04 per cent Ca and 0.70 per cent P. The Ca:P ratio was 1:17.5. The diet was supplied with vitamin D_2 600 I.U. per 100 g diet.

The normal calcium diet consisted of the low calcium test diet supplemented with $CaCO_3$, this diet thus containing a total amount of 1.2 per cent Ca and 0.70 per cent P. The Ca:P ratio was 1:0.53.

Diets and deionized water were given *ad libitum*.

Parathyroidectomy

In the two series, a total of 95 animals had their parathyroids removed by electro-coagulation with a fine diathermy needle under light ether anaesthesia (PTE animals). The thyroid gland and the parathyroids were exposed using a dissection microscope and under sterile operative conditions. The parathyroids were visualized after the surface of the thyroid gland had been brushed with 1:1000 adrenaline solution as recommended by *Bralin* (1945). Completeness of removal was checked by analyses of plasma calcium (see Plasma Sampling). Only animals which showed a postoperative fall in the plasma calcium level to below 4.1 mEq./l were accepted as PTE animals. This limit has been used in most previous studies on PTE rats (cf. *Johansson & Segerström* 1972).

Experimental animals. PTE animals were divided into the following groups of the short-term series:

PTE —Ca	eight PTE rats supplied the low calcium diet.
PTE +Ca	ten PTE rats supplied the normal calcium diet showing a consistently reduced plasma calcium level.
PTE_x +Ca	six PTE rats given the normal calcium diet showing a normalization of the plasma calcium level after an initial reduction.
PTE_{NV} +Ca	in six of the operated animals the effect of PTE could not be verified since no reduction of the plasma calcium level could be observed.

Obtained from Møllegaard Hansen Avlslaboratorier A/S, Ejby Denmark.

** Here and in the following text the figures calculated on a dry weight basis are given.

Obtained from Ewos Co., Södertälje Sweden.

Intact animals were randomly divided into the following groups

—Ca five animals supplied the low calcium diet.
+Ca five animals given the normal calcium diet thus constituting the control group.

Body weights Food Consumption and Stools

All the animals were weighed at several intervals during the experimental period. Food consumption and stool production were determined after 4 weeks from the start of the experiment in the intact animals fed the normal calcium diet and in the PTE animals supplied the low and normal calcium diet, respectively. For this purpose, the animals were kept in metabolic cages during 8 days and the weights of the consumed food were carefully recorded by daily measurements of the weights of the supplied and not consumed food, respectively. The stools were collected daily in a pre-weighed bottle with a screw-lock and the weights of the specimens pooled for each animal during the 8 days were thereafter determined on a Mettler balance.

Plasma Sampling

From each animal, approximately 0.5–1.0 ml of blood was taken by cardiac puncture under light ether anaesthesia 2 days, 4 and 6 weeks after the start of the experiment and 4 24 48 and 72 hours before sacrifice of the animal. Finally blood was collected at the time of sacrifice which was performed by bleeding the animal to death from the femoral artery under ether anaesthesia. The collected blood was centrifuged at 3,000 r.p.m. for 15 minutes and the supernatant plasma was pipetted into acid washed test tubes and kept at 20 pending subsequent analyses.

Determination of Plasma Calcium

This was performed by atomic absorption spectrophotometry after precipitation of proteins with 20 per cent trichloro-acetic acid and addition of $SrCl_2$ for depression of the phosphorus interference according to *Willis* (1961) The absorption was read in a Unicam SP 90 spectrophotometer * The coefficient of variation for these analyses was less than 1 per cent.

Determination of Moisture Organic and In-Organic Matter of the Tibia

After careful dissection from soft tissues and periosteum, the right tibia was placed in a weighing container and the wet weight was immediately recorded in Mettler balance with an accuracy of 0.05 mg. All specimens within the series were thereupon dried simultaneously at 50° C for 48 hours and the weight of the bones was then recorded. After ashing the bones in a muffle furnace at 700° C for 16 hours, the weight of the ashes was recorded.

Preparation of Undecalcified Bone Sections

The right femur of all the rats was freed of the surrounding soft tissues and then divided exactly in the middle of the diaphysis and perpendicularly to the long axis of the bone, using a fine band saw The distal part of the bone was then fixed and dehydrated in 6 changes of 96 per cent alcohol for 6 days. The specimens were embedded in methacrylate as described earlier (*Larsson* 1969) From the diaphyseal end of the bone specimen a section approximately 0.5 mm thick was cut exactly perpendicularly to the long axis of the bone. Another section was cut in the same way from the distal metaphysis of the femur just proximally to the level where the articular cartilage facing the patella ended. All sections were ground on a Knuth-Rotor grinder to a thickness of approximately 80 μ with water as a lubricant and using Silicone-Carbide papers Nos. 220 and 600

Microradiography

This was performed using a Siemens tube (AGW 3 8, with a 1 mm² tungsten target and a 0.1 mm thick beryllium window) at 15 kV the sections were placed in direct contact with Kodak spectroscopic plates 649-0.

Measurement of the Cortical Thickness of the Mid-Shaft of the Femur

This was performed on photo-negatives of a magnification of 10× The thickness of the two cortices was determined both along the long diameter of the cross-section of the bone and along the short diameter

Morphometric Analyses of the Relative Amount of Spongious Bone Tissue

This was determined by the point sampling method according to *Chalkley* (1943) using an integrating eyepiece (Zeiss I) equipped with 25 reference points. Seven measurements were made at a linear magnification of 62× on microradiographs from the metaphyseal bone sections obtained as described above. The areas were chosen in a standardized way in order that all the spongious bone and no cortical bone would be examined on each section.

Determination of the Calcium Accretion Rate for the Tibia

The accretion rates for the whole tibia, expressed as mg calcium/hour/mg calcium of the bone were calculated according to *Bauer et al.* (1955)

* Unicam Instr. Ltd., London, England.

dihydroxycholecalciferol in response to changes in calcium intake and plasma calcium level (*Garabedian et al.* 1972)

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- PTE_{xx} +Ca: in six of the operated animals the effect of PTE could not be verified since no reduction of the plasma calcium level could be observed.

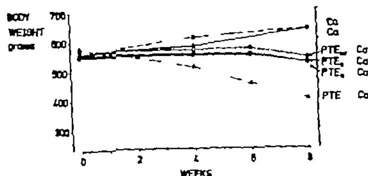
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* Obtained from Møllegaard Hansens A laboratorier A/S Ejby Denmark.

** Here and in the following text the figures calculated on a dry weight basis are given.

* Obtained from Ewos Co., Södertälje, Sweden.

Fig 1 Mean body weights of the normal control group and the various experimental groups during the experimental period of 8 weeks (+Ca = normal dietary calcium —Ca = low dietary calcium PTE = parathyroidectomy PTE_N = PTE animals showing a normalized plasma calcium level PTE_R = PTE animals showing a reduced plasma calcium level PTE_{NV} = PTE animals in which the effect of PTE could not be verified)



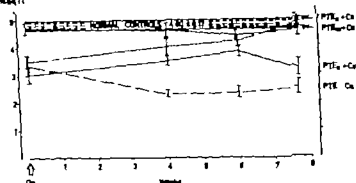


Fig 2 Plasma calcium of the normal control and the same groups of experimental animals as in Fig 1 The bars represent the standard error of the mean. Analyses were made at 2 days and at 4 6 and 8 weeks after the start of the experiment. The values obtained in the two groups of animals which were kept on normal and low dietary calcium, respectively did not differ significantly

ma calcium values varying between 2.65 and 4 0 mEq /l after 4 6 and 8 weeks.

At a low intake of calcium in the diet all PTE animals showed very low plasma calcium values varying between 2.05 and 2.95 mEq./l after 4 6 and 8 weeks. No tendency towards a normalization of the values was observed during this period.

Short Term Series

Body Weight

Intact rats showed an increase by 12 per cent in body weight during the experimental period (Fig. 1) This increase was observed both at a low and at a normal intake of calcium in the diet. PTE animals showed a tendency towards a slight reduction in body weight when they were kept on a normal dietary calcium, no matter whether the animal were hypocalcaemic or they showed a normalized plasma calcium. At a low intake of calcium in the diet, PTE animals showed a reduction in their body weight by approximately 30 per cent.

Composition of the Tibia

The contents of moisture and organic matter of the whole tibia showed no significant differences between the various experimental groups and the normal controls. The inorganic contents (Table 1) showed a slight tendency towards higher values in all PTE animals compared with the normal controls. These differences were not significant however

Cortical Thickness of the Mid Shaft of the Femur

Compared with the normal control animals, calcium-deficient intact animals showed significantly lower values while no significant difference was found in the case of calcium-

TABLE 1 *The Inorganic Matter of the Tibia, the Cortical Thickness of the Midshaft of the Femur and the Number of Spongious Bone "Hits" in the Normal Control Group and the Various Experimental Groups*

Group	Tibia	Femur	
	Per cent inorganic matter	Cortical thickness, mm	Spongious bone "hits"
Intact controls, + Ca	47.46 ± 0.346	2.23 ± 0.019	33.80 ± 5.063
Intact animals, —Ca	47.49 ± 0.476	2.02 ± 0.078	22.20 ± 4.029
PTE animals, + Ca	48.43 ± 0.529	2.36 ± 0.111	36.56 ± 4.024
PTE animals, —Ca	48.28 ± 0.435	2.17 ± 0.106	37.00 ± 6.449
PTE_X animals, + Ca	48.35 ± 0.486	2.34 ± 0.139	32.17 ± 6.139
PTE_{XT} animals, + Ca	48.21 ± 0.425	2.12 ± 0.487	38.66 ± 3.648

Mean values and standard errors are given. Note the reduction in cortical and spongious bone in intact animals kept on the low calcium intake but not in PTE animals kept on the same level of dietary calcium.

deficient PTE animals (Table 1). The obtained values for PTE animals supplied the normal calcium diet did not differ significantly from those of the intact control rats.

Morphometric Analyses of the Relative Amount of Spongious Bone Tissue

Measurements by point sampling of the spongious bone of the distal femur metaphysis gave lower values for intact rats fed the low calcium diet compared with those obtained for the normal control rats, the difference being almost significant (Table 1). There was no significant difference between the values obtained for the rats of the normal control group and those of the various groups of PTE animals which showed similar values regardless of the level of dietary calcium. The values of the calcium-deficient intact rats were significantly lower than those obtained for PTE animals supplied the normal calcium diet showing reduced plasma calcium level. In comparison with the values of the calcium-deficient PTE animals, lower values were obtained for calcium-deficient intact rats, the difference being almost significant ($0.05 < p < 0.10$).

Microradiography

Microradiographs of undecalcified cross sections of the distal femur metaphysis showed that the cortical and trabecular bone in the normal control rats was quite evenly mineralized (Figs. 3 and 4). In the intact animals maintained on the low calcium diet for 8 weeks, the microradiographs were of an *appearance similar to those in the control* group with the exception that the bone trabeculae were numerically fewer and considerably thinner (Figs. 5 and 6). There was *no difference in the degree of mineralization* of the cortical and the spongious bone. The microradiographs obtained from the PTE animals supplied the normal calcium diet had the same appearance as those of the normal controls (Figs. 7 and 8). The same observation applied also to the PTE animals fed the low calcium diet (Figs. 9 and 10).

Fig. 3–10. Microradiographs of undecalcified cross sections of the distal femur metaphysis at linear magnifications of 20 × and 50 ×. At the end of the experimental period of 8 weeks there was an obvious difference between intact animals kept on normal (Figs. 3 and 4) and a low dietary calcium (Figs. 5 and 6) in that the latter showed the occurrence of osteoporosis with loss of spongious bone, the remaining bone showing the normal degree of mineralization. In contrast, PTE animals showed no change in the amount of spongious or cortical bone either at a normal (Figs. 7 and 8) or at a low calcium intake (Figs. 9 and 10) and the mineralization of the bone was normal.

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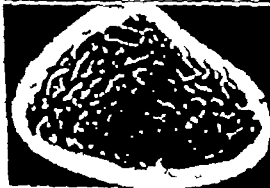
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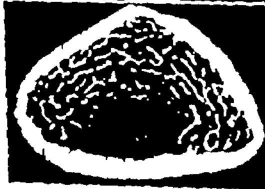
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TABLE 2. *Calcium Accretion Rate of the Tibia in Control and Experimental Groups*

Group		mg Ca/h./mg Ca of the tibia × 10^{-7}
Intact controls,	+Ca	5.25 ± 0.184
Intact animals,	—Ca	6.64 ± 0.334
PTE animals,	+Ca	4.73 ± 0.459
PTE animals,	—Ca	4.45 ± 0.248
PTE_N animals,	+Ca	6.19 ± 0.421
PTE_{NT} animals,	+Ca	4.61 ± 0.395

Note the increased accretion rate in intact animals kept on the low calcium intake and the reduced rate in PTE animals kept on the same level of dietary calcium.

Calcium Accretion Rate by Bone

The accretion rate of the tibia showed significantly higher values for the intact rats fed the low calcium diet compared with those of the normal control animals (Table 2). In contrast, PTE rats fed the low calcium diet showed significantly lower values than those of the normal controls. As regards PTE animals supplied the normal calcium diet, values were obtained which showed a tendency to be lower in the case of rats having a reduced plasma calcium and higher in those showing a normalized plasma calcium compared with the values of the normal controls. The accretion rates applying to the PTE rats kept on the normal calcium intake and showing a consistently reduced plasma calcium were significantly lower than those obtained in the PTE animals which had normalized their plasma calcium.

Effects of PTE on the Thyroid Gland

At histological examination of serial sections of the thyroid gland, remaining parathyroids were found in only 2 out of a total of 95 PTE animals. These animals which did not show a reduction of their plasma calcium were included into the PTE_{N} group and had been supplied the normal calcium diet. In the other 4 animals of the PTE_{N} group, no remaining parathyroid glands were found within the thyroid gland.

Only scar tissue remained at the site of the parathyroids after their removal by diathermy involving only a small region of the thyroid gland. Otherwise the thyroid had quite a normal histological appearance except for one animal which had a small colloidal adenoma.

Effects of PTE on the Adrenal Glands

At determination of the wet weight of the two glands and at morphometric analysis

TABLE 3. *The Wet Weight of the Adrenal Glands (Mean Weight of the Two Glands) and the Relative Thickness of the Adrenal Cortex in Normal Control Rats and Experimental Rats*

Group		Wet weight of the adrenal glands, mg	Relative thickness of the adrenal cortex, per cent
Intact controls,	+Ca	27.91 ± 0.639	36.20 ± 2.805
Intact animals,	—Ca	29.72 ± 1.190	44.26 ± 2.288
PTE animals,	+Ca	29.16 ± 1.246	40.30 ± 1.257
PTE animals,	—Ca	29.50 ± 1.763	40.51 ± 1.-92
PTE_N animals,	+Ca	31.03 ± 2.032	44.02 ± 3.437
PTE_{NT} animals,	+Ca	30.05 ± 1.216	40.48 ± 0.894

There were no statistically significant differences between the groups.

Fig. 11–14. Photomicrographs of the adrenal glands and the adrenal cortex stained with Sudan Black B in a normal control rat (above) and a PTE animal fed the low calcium diet (below). In the latter animal there was some evidence of hyperactivity of the adrenal cortex that showed relatively abundant contents of lipids, the glomerular zone being less well-demarcated from the fasciculate zone than normal. Magnification 20× and 50×

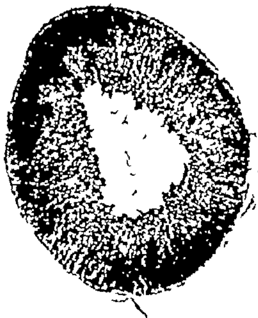


Fig 11

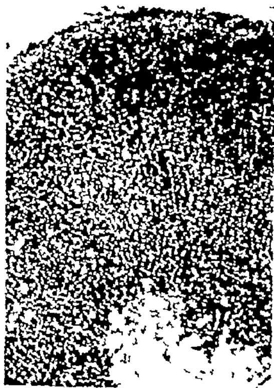


Fig 12

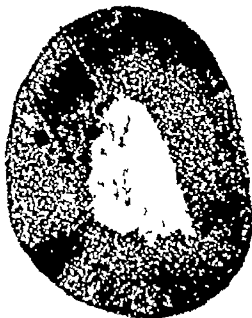


Fig 13



Fig 14

of the relative thickness of the adrenal cortex no significant differences between the normal control group and the various experimental groups were found (Table 3) Microscopical examination of frozen sections stained with Sudan Black B showed the normal adrenal cortex to be relatively rich in lipids, the glomerular zone appearing well-demarcated from the fasciculate zone (Figs. 11 and 12) In most of the experimental animals no noteworthy changes were found. However in the group of calcium-deficient animals which showed a significant reduction in body weight, minor histological changes of the adrenal cortex were observed. In some of these animals the glomerular zone was not well-demarcated from the fasciculate zone and both zones appeared to have relatively abundant contents of lipids (Figs. 13 and 14) *These changes were found in only 3 animals* of this group while no changes were observed in the remaining 4 animals.

DISCUSSION

In the present investigation, selective parathyroidectomy was performed using a careful electro-coagulation technique in order not to influence the thyroid function and the pro- tion of thyro-calcitonin. The thyroid gland was mainly left intact *in situ* Supernumerary parathyroid glands in the neck present in 30 per cent have been reported to be hormonally active although inadequate for maintaining a normal plasma calcium level after parathyroidectomy (*Caswell & Fennell* 1970) Therefore, a defined decrease of the plasma calcium level was chosen as criterion for an absence of parathyroid function. A postoperative decrease of the plasma calcium level to 4 1 mEq /l or less, as used by *Johansson & Segerström* (1972) for the same strain of rats, constituted our criterion for a verified parathyroidectomy In the present study a verified parathyroidectomy was achieved in as much 86 per cent of the rats fed the normal c um diet.

Histological examin. of the serial-sectioned thyroid gland re -d that one of the parathyroids remained intact in two animals while no parathyroid gland was found in the other rats. Thus in ten out of a total of seventy-two surviving parathyroidectomized rats (13.9 per cent) the maintenance or normalization of the plasma calcium level could be due to accessory parathyroid glands located in the mediastinum. In addition, a certain number of animals could have accessory parathyroid glands with a hormonal activity that was inadequate for a normalization of their plasma calcium. This will be subjected to further studies which will be reported elsewhere.

The efficiency of maintaining the plasma calcium level after selective parathyroidectomy was found to be dependent upon the level of dietary calcium intake. At a normal calcium intake six animals (17 per cent) *showed a normalization of their plasma calcium* level within 8 weeks. Furthermore, the six animals which did not show a postoperative reduction of their plasma calcium were all fed the normal calcium diet. At a low calcium intake, all parathyroidectomized rats showed a postoperative reduction of plasma calcium with no tendency towards a normalization later At a normal calcium intake, the group of parathyroidectomized rats with a reduced plasma calcium showed a mean reduction of the plasma calcium level by 33 per cent at the end of the experimental period while at a low calcium intake the corresponding figure was 48 per cent. Thus, selective parathyroidectomy resulted in an inability of adaptation to a low calcium intake, viz. an inability to maintain a normal plasma calcium level.

The significant reduction in body weight observed in the group of parathyroidectomized rats fed the low calcium diet was apparently due to less food consumption than normal. This was evident from the determinations of the amounts of food intake and the stools. Of seven animals in this group three showed quite a small food consumption. In these rats, an indication of stress was obtained from histological examination of the adrenal glands that revealed an abundance

of lipids in the slightly thickened fasciculate zone and a glomerular zone less sharply demarcated than normal. Generally there was no evident effect of the various treatments upon the adrenal cortex. The weight of the adrenal glands showed no significant change nor did the thickness of the adrenal cortex as determined by morphometric analyses of histological freeze-sections. The adrenal cortex had a normal histological appearance except for the three animals showing a stress reaction. Thus there was no indication of a possible secondary effect of the removal of the parathyroids and the instituted low calcium diet upon the functional state of the adrenal cortex. It could be considered, therefore, that the reported changes of calcium metabolism and bone were primarily caused by the induced changes of endogenous parathyroid hormone secretion and the dietary calcium intake and not by any major influence exerted by the activity of the thyroid and adrenal glands.

The osteoporotic bone changes in the calcium-deficient intact animals described in the present study were in agreement with those reported by *Larsson* (1969). Morphometric analysis of the bone mass on microradiographs of standardized undecalcified bone sections was found to be a more sensitive method for the determination of loss of bone tissue than the analysis of the contents of inorganic matter of a whole bone. While osteoporosis resulted to a significant degree in the intact calcium-deficient animals this did not occur in the parathyroidectomized rats fed the same low calcium diet. Thus, selective parathyroidectomy inhibited the occurrence of calcium-deficiency osteoporosis in the adult rat. This demonstrates the fundamental role of the parathyroids for the adaptation to a low calcium intake since the mobilization of skeletal calcium to the blood is a very important adaptatory mechanism (*Larsson* 1969). Increased parathyroid activity as indicated by the occurrence of parathyroid hyperplasia (*Sevastikoglou* & *Larsson* 1972) is certainly responsible for the increased loss of bone tissue in calcium-deficient animals in which the plasma calcium level is maintained at a normal or slightly subnormal level at the expense of the bone tissue (*Larsson* 1969). This is in accordance with reports of studies of other species as cats (*Jowsey* & *Gershon-Cohen* 1964, *Jowsey* & *Raisz* 1968) and dogs (*Saville* & *Krook* 1968).

While in the present investigation a protective effect of selective parathyroidectomy against the occurrence of calcium-deficiency osteoporosis was found, calcium-deficient thyroparathyroidectomized adult rats of the same strain have been reported not to develop this kind of osteoporosis (*Sevastikoglou* 1972). In that situation, the occurrence of osteoporosis might be secondary to nutritional disturbances induced by the removal of the thyroid gland rather than to a possible increased activity of vitamin D secondary to the low calcium intake. The active metabolite of vitamin D, 1,25-dihydroxycholecalciferol, has been reported to be a potent stimulator of bone resorption in tissue culture (*Raisz et al.* 1972). However thyroparathyroidectomy of rats on a diet low in calcium has recently been shown to result in a reduced production of this active metabolite of vitamin D to negligible levels (*Garabedian et al.* 1972). The latter possibility therefore seems less likely as an explanation of the occurrence of osteoporosis in calcium-deficient thyroparathyroidectomized rats and thus, secondary nutritional factors are probably of more decisive importance.

The increased calcium accretion rate by bone recorded in the calcium-deficient intact animals of the present study seems to be secondary to the increased mobilization of skeletal calcium caused by the increased parathyroid activity. Consequently a reduced calcium accretion rate was found in the parathyroidectomized animals both at a normal and at a low intake of dietary calcium.

At present, the parathyroids are ascribed a possible role in regulating the metabolism of vitamin D (*Omdahl* & *DeLuca* 1973). An increased synthesis of the metabolically active form of vitamin D, 1,25-dihydroxycholecalciferol, in response to a reduction in

calcium intake and plasma calcium level has been reported (*Garabedian et al.* 1972). However no disturbance of the intestinal calcium absorption was found after selective parathyroidectomy in the same animals used in the present study indicating that parathyroid hormone has no direct regulatory influence on the metabolism of vitamin D (*Ahlgren* & *Larsson* 1975). In view of these data, it appears that the active metabolite of vitamin D stimulates the intestinal calcium absorption in the absence of the parathyroids but not the mobilization of skeletal calcium necessary for the adaptation to a low calcium intake. While intact animals developed calcium deficiency osteoporosis, parathyroidectomized animals did not.

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THE ROLE OF THE PARATHYROIDS FOR THE ADAPTATION TO A LOW CALCIUM INTAKE

2 The Short Term Effect of Parathyroidectomy on the Adaptation to a Low Calcium Intake in Adult Rats with Special Reference to Calcium Metabolism

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The effects of selective parathyroidectomy on the adaptation to a low calcium intake were studied in one-year-old male rats. Parathyroidectomized animals showed increased net absorption of intestinal calcium compared to that of intact animals both at a normal and at a low intake of dietary calcium. The difference was highly significant on the low level of dietary calcium. In the parathyroidectomized animals, significantly higher values were found in the group of rats fed the low calcium diet than in those supplied the calcium supplemented diet. Urinary calcium excretion was reduced because of the reduced plasma calcium level. While there was no disturbance of intestinal calcium absorption, selective parathyroidectomy resulted in reduced ability to mobilize skeletal calcium as shown in a previous study This was found to be necessary for the adaptation to a low calcium intake in the adult animal.

Key words: Calcium deficiency; Calcium metabolism Parathyroidectomy Osteoporosis.

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The parathyroids, besides vitamin D appear to be of primary importance for the adaptation to a low calcium intake in the adult rat as demonstrated by the occurrence of parathyroid hyperplasia upon reduction of the dietary calcium intake (cf *Sevastikoglou & Larsson* 1972) Through the influence of parathyroid hormone skeletal calcium reserves must become mobilized with resulting osteoporosis (cf. *Larsson* 1969) After parathyroidectomy there is an inability to mobilize skeletal calcium to the blood which is necessary for adaptation and no osteoporosis occurs (*Larsson & Ahlgren* 1975) The present investigation was undertaken in order to obtain information about the effects of parathyroidectomy on the other mechanisms involved in the adaptation to a low calcium intake i.e calcium absorption and excretion.

Vitamin D has been shown to be essential for the adaptation to a reduced calcium intake in young rats by increasing intestinal calcium absorption (*Nicolaysen et al.* 1953) Recently the parathyroids have been suggested to act as a major regulator of calcium absorption by regulating the synthesis of the metabolically active form of vitamin D 1,25-

dihydroxycholecalciferol, in response to changes in calcium intake and plasma calcium level (*Boyle et al.* 1971 *Garabedian et al* 1972, *Galante et al* 1972) However previous studies designed to answer the question whether or not endogenous parathyroid hormone has an influence on gastro-intestinal absorption of calcium have not completely resolved this problem.

At a normal intake of dietary calcium, parathyroidectomy has been reported in some studies (*Cramer et al* 1961 *Lammon et al* 1970 *Toverud* 1964) to result in inhibited net absorption of calcium while several other studies (*Gran* 1960 *Clark & Rivera-Cordero* 1971) indicate that there is no effect on the calcium absorption. In fact, increased calcium absorption after parathyroidectomy has also been reported and decreased calcium absorption after the administration of para thyroid extract to parathyroidectomized rats (*Clark & Rivera-Cordero* 1971)

At a low intake of calcium in the diet, parathyroidectomized rats have been reported to absorb less calcium than intact controls (*Shah & Draper* 1966) but this finding has been the subject of disagreement (*Lammon et al.* 1970) Recently it has been reported that there is no difference in cal cium absorption in intact and parathyroid ectomized rats at a normal intake of calcium while at low levels of dietary calcium para thyroidectomized rats absorbed less calcium than their intact controls (*Clark & Rivera-Cordero* 1973) Calcium absorption studies based on preparations *in vitro* or isolated intestinal segments *in situ* have not yielded conclusive results (*Rasmussen* 1959 *Dowdle* 1960 *Lifshitz* 1961 *Winter et al.* 1970)

In the present investigation the effects of selective parathyroidectomy upon plasma calcium and inorganic phosphate, urinary excretion of calcium and inorganic phosphate and net absorption of intestinal calcium were studied in adult ats at normal and low levels of dietary calcium Furthermore, the plasma ^{45}Ca activities a elimination rates were analysed after adm istration of the isotope by stomach tube an lso faecal and urinary ^{45}Ca activities as well as the incorporation of ^{45}Ca by bone.

MATERIAL AND METHODS

The experimental material is the same as that used in the previous investigation (*Larsson & Ahlgren* 1975) A total of 40 sterile-bred 12–12½ months old male rats of the Sprague-Dawley strain* were used. All animals had been bred on a standard laboratory ration described by *Eggum & Mollegaard-Hansen* (1967)

Housing

The animals were housed five to each cage at a temperature of 18–20 without access to direct sunlight. For studies of the calcium metabolism the animals were kept individually in metabolic cages for a period of 3 days before sacrifice.

Experimental Diets

The low calcium diet of General Biochemical Inc., Chagrin Falls, Ohio, was used as a basic ration. The composition and ingredients of this diet (GBI Bulletin D-15) has been described in detail elsewhere (*Larsson* 1969) Chemical analyses showed that the diet contained 0 04 per cent Ca and 0 70 per cent P The Ca P ratio was 1 17.5 The diet was supplied with vitamin D_3 600 I U per 100 g diet.

The normal calcium diet consisted of the low calcium test diet supplemented with $CaCO_3$, this diet thus containing a total amount of 1.. per cent Ca and 0.70 per cent P The Ca P ratio was 1·0.58.

Diets** and de-ionized water were given *ad libitum*.

P rathyroidectomy

This was performed by electro-coagulation with a fine diathermy needle under light ether anaesthesia as described previously (*Larsson & Ahlgren* 1975) Completeness of removal was checked by analyses of plasma calcium. Only animals which showed a postoperative fall in the plasma calcium level to below 4 1 mEq./l were accepted as PTE animals. This criterion has been used in previous studies on PTE rats (*Johansson & Segerström* 1972)

Experimental Animals

The PTE animals were divided into the following groups

Sterile-bred rats obtained from Møllegaard Hansen s Avlslaboratorier A/S Ejby Denmark.
** Obtained from Ewos Co Södertälje, Sweden.

PTE —Ca eight PTE rats supplied the low calcium diet.

PTE +Ca ten PTE rats supplied the normal calcium diet showing a consistently reduced plasma calcium level.

PTE_N +Ca six PTE rats given the normal calcium diet showing a normalization of the plasma calcium level after an initial reduction.

PTE_{NV} +Ca; in six of the operated animals the effect of PTE could not be verified since no reduction of the plasma calcium level could be observed.

Intact animals were randomly divided into the following groups

—Ca five animals supplied the low calcium diet.
+Ca five animals given the normal calcium diet thus constituting the control group

Experimental Period

All animals received their respective experimental diet from the day of parathyroidectomy and were observed for an experimental period of 8 weeks as were also the intact control animals.

Isotope Administration

Seventy two hours before sacrifice, each animal received 20 microcuries of ^{45}Ca in 1.0 ml physiological saline solution from a single batch of $^{45}CaCl_2$ for the whole series. The isotope solution was administered by stomach tube under light ether anaesthesia. The technique of placing a fine polyethylene catheter into the stomach of the rat had been trained in before on a certain number of animals with control of the exact position of the tip of the catheter on roentgenograms.

Plasma Sampling

From each animal approximately 0.5–1.0 ml of blood was taken by cardiac puncture under light ether anaesthesia at 2 days, 4 and 6 weeks after the start of the experiment and at 4 24 48 and 72 hours before sacrifice of the animal. Finally blood was collected from all animals at the time of sacrifice which was performed by bleeding the animal to death from the femoral artery under ether anaesthesia. The collected blood was centrifuged at 3,000 r.p.m. for 15 minutes and the supernatant plasma was pipetted into acid washed test tubes and kept at –20 pending subsequent analyses

Calcium chloride in aqueous solution 2–10 mCi/mg Ca, CES. 2, The Radiochemical Centre, Amersham, England.

Determination of Plasma Calcium and Magnesium

This was performed by atomic absorption spectrophotometry after precipitation of proteins with 20 per cent trichloroacetic acid and addition of $SrCl_2$ for depression of the phosphorus interference as described by *Willis* (1961) The absorption was read in a Unicam SP 90 spectrophotometer* The coefficient of variation for these analyses was less than 1 per cent.

Determination of Inorganic Phosphate of Plasma

This was performed with the aid of a Technicon auto-analyzer** The coefficient of variation for these analyses was within 1.5 per cent.

Determination of Urinary Calcium Magnesium and Inorganic Phosphate

Calcium and magnesium were determined by atomic absorption spectrophotometry after precipitation of proteins with 20 per cent trichloroacetic acid and addition of $SrCl_2$ for depression of the phosphorus interference as recommended by *Willis* (1961) The absorption was read in a Unicam SP 90 spectrophotometer Inorganic phosphate was determined with the aid of a Technicon autoanalyzer The coefficients of variation for these analyses were the same as those of the corresponding analyses on plasma.

Determination of Faecal Calcium and Magnesium

This was performed on the ashed and dissolved faecal samples by the same methods as those used for the corresponding analyses of the urinary samples.

Determination of the ^{45}Ca Activity

The radioactivity of plasma and urine was determined directly by adding 0.2 and 0.5 ml of the samples to the liquid scintillator composed of 2,5-Diphenyloxazole (PPO) 1.0 g, 1 4-bis-2 (4-Methyl-5-Phenyloxazolyl)—Benzene (POPOP) 0.3 g Naphtalene 100.0 g Dioxane 1 litre and thixotrophic gel powder (CAB-O-SIL) to saturation. The radioactivity of the tibia was determined after dissolving the ashed bone in 10.0 ml of 1.2 N hydrochloric acid and adjusting the volume to 100.0 ml by adding re-distilled water. Aliquots of 2.0 ml were added to the liquid scintillator Counting was carried out in a Packard Tri-Carb Liquid Scintillation Spectrometer*** as described in detail previously (*Larsen* 1969)

* Unicam Instr Ltd London, England.
** Technicon Instrument Corp. Ardsley N Y 10502 USA.
*** Model 314 EX 2, Packard Instruments Co., La Grange, Ill., USA.

Statistical T eatment of Data

Data applying to the different groups were compared in pairs and subjected to the Student's t-test. The hypothesis of no difference between groups was tested with a 5 per cent significance level.

RESULTS

Plasma Calcium
Faecal and Urinary Calcium

As regards intact animals, plasma calcium showed no significant difference between rats fed the low calcium diet and those supplied the calcium supplemented diet (Fig 1)

Six PTE animals did not show any post operative reduction of the plasma calcium level and consequently the effect of PTE could not be verified in these animals all of which were supplied the calcium supplemented diet (PTE_{NV} + Ca) Six of the PTE rats given the normal calcium diet showed a normalization of the plasma calcium level after an initial reduction whereas ten animals showed a consistently reduced level. All of the eight PTE rats which were supplied the low calcium diet showed consistently reduced plasma calcium values with no tendency towards a normalization. These animals showed significantly lower plasma calcium than the PTE animals fed the calcium supplemented diet the mean reduction amounting to 47.9 and 32.3 per cent, respectively

Faecal calcium showed significantly lower values in the animals fed the low calcium diet than in those fed the calcium supplemented diet, as was expected. There was no significant difference in faecal calcium within the groups of rats fed the low and the normal calcium diet, respectively

Urinary calcium was significantly lower in all experimental groups compared with that in the normal control group. There were no significant differences between the various experimental groups

The ⁴⁵Ca Activity f Plasma, Faeces Urine and Bone

The plasma ⁴⁵Ca a tivity expressed in per cent of administered d se per mg plasma cal

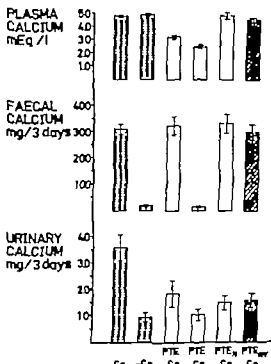


Fig 1 Plasma calcium, faecal and urinary calcium of the normal control group and the various experimental groups (+Ca = normal dietary calcium —Ca = low dietary calcium PTE = parathyroidectomy PTE_N = PTE animals showing a normalized plasma calcium level; PTE_{NV} = PTE animals in which the effect of PTE could not be verified) The bars represent the standard error of the mean.

cium was significantly higher in the calcium-deficient intact animals than in the normal controls fed the calcium supplemented diet (Fig 2) PTE animals showed significantly higher plasma ⁴⁵Ca-activity than intact animals both at a normal and at a low intake of dietary calcium. The highest values were obtained in the group of PTE animals fed the low calcium diet. The group of PTE animals with normalized plasma calcium showed higher values than those of the normal control group the difference being almost significant. This was also found to apply to the group of rats with a non-verified parathyroidectomy

The faecal ⁴⁵Ca-activity expressed as per cent of administered dose showed lower values in all experimental groups compared with those of the normal control group (Fig

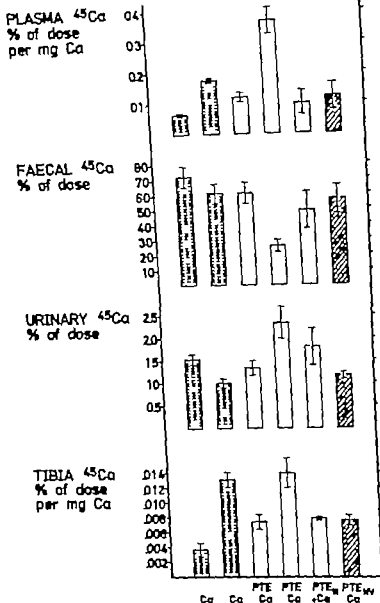


Fig 2 The ⁴⁵Ca-activity of plasma, faeces, urine and bone of the same groups of animals as in Fig. 1 The isotope was administered by stomach tube 72 hours before sacrifice. Note the higher ⁴⁵Ca-activity of plasma and bone with lower ⁴⁵Ca-activity of faeces in PTE animals than in intact animals both at a normal and at a low intake of dietary calcium.

2) However only the group of PTE animals fed the low calcium diet showed significantly reduced values.

The urinary ⁴⁵Ca-activity expressed in per cent of dose, was significantly lower in calcium-deficient intact animals than in the normal controls (Fig. 2) The PTE animals showed no significant difference from normal at a normal intake of calcium. At a low calcium intake, however the values obtained in the PTE animals were significantly higher than those obtained in intact animals.

The ⁴⁵Ca-activity of the tibia, expressed in per cent of dose per mg calcium of the bone, showed significantly higher values in all experimental groups than in the normal control group (Fig 2) At a low calcium intake, highly increased values were obtained both in the PTE animals and in the intact animals. There was no significant difference between

the values of these two groups of rats. At a normal level of dietary calcium, the values obtained in the PTE animals were signifi cantly higher than those obtained in the normal control animals. In fact, the increase in the two groups of PTE animals showing either reduced or normalized plasma calcium was found to be the same as that in the group of rats in which the effect of parathyroid ectomy could not be verified.

The Plasma ^{45}Ca Activity at Different Periods after the ^{45}Ca Administration

The ^{45}Ca activity of plasma expressed in per cent of dose per mg calcium of the plasma, showed values which were considerably higher in all experimental groups than in the normal control group (Fig 3) At a normal calcium intake, values in the PTE animals were found to be approximately *twice* as high as those in the normal control animals. This was found to apply equally well to PTE animals with reduced plasma calcium and to those with normalized plasma calcium, both groups of rats showing a tendency towards a more rapid elimination of the ^{45}Ca-activity than the intact animals. Animals in which the effect of PTE could not be verified showed also higher values than those of the normal control animals.

At a low calcium intake, the level of the plasma ^{45}Ca-activity was almost three times higher in the intact animals and almost six times higher in the PTE animals than in the normal control rats fed the calcium supple mented diet.

Intestinal Net Absorption of Calcium

In the intact animals, higher values were obtained in the group of rats fed the low *calcium diet than in those fed the calcium*

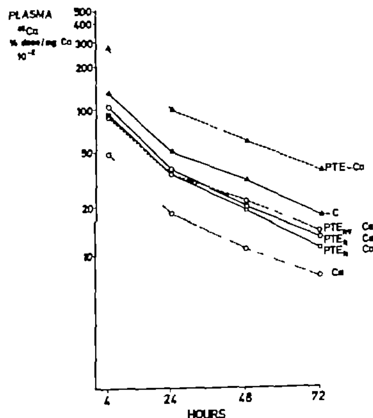


Fig 3 The plasma ^{45}Ca-activity at different periods after the administration of the isotope by stomach tube. Note the higher activity in PTE animals compared w th intact animals, both at normal and at a low intake f d etary calcium. *This differer was* found also when differ- m body weight were taken n sideration.

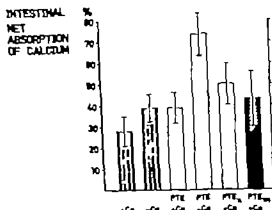


Fig 4 Intestinal net absorption of calcium. At a low intake of calcium, the values in PTE animals were significantly higher than those in intact animals. In the PTE animals, significantly higher values were obtained on the low level of dietary calcium than on the normal intake of calcium.

supplemented diet, but the difference was not statistically significant due to the variation of the values within the groups (Fig 4) At a normal level of dietary calcium, the PTE animals showed higher values than those of the normal control rats although the difference was not significant. At a low intake of calcium, higher values were obtained in the PTE animals than in the intact animals, the difference being highly significant. In the PTE animals, significantly higher values were found in the group of rats fed the low calcium diet than in those supplied the calcium supplemented diet.

Plasma Magnesium
Faecal and Urinary Magnesium

Plasma magnesium showed no significant difference between the values of the calcium-deficient intact animals and those of the normal controls (Fig. 5) At a normal calcium intake the PTE animals showed values which did not differ from those of the intact animals. At a low calcium intake, however significantly lower values were obtained for the PTE animals than for the intact animals. As regards the remaining groups of rats, no significant changes were found.

Values of faecal magnesium were found to be lower in the calcium-deficient intact animals than in the normal controls, the difference being almost significant (Fig 5) At a normal calcium intake, the values of the PTE animals showed no significant difference from those of the intact animals. At a low calcium intake, however significantly higher values were obtained in the PTE animals than in the intact animals. The calcium-deficient PTE animals showed also significantly higher values than those of the PTE animals fed the calcium supplemented diet. The remaining groups of rats showed no significant differences from normal.

Values of urinary magnesium were found to be higher in the calcium-deficient intact animals than in the normal controls, the difference being almost significant (Fig 5) The remaining groups of animals showed no changes from normal.

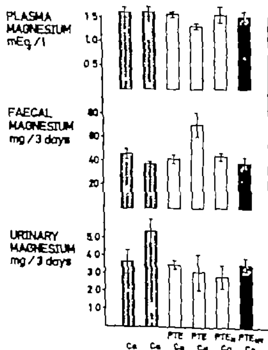


Fig 5 Plasma magnesium, faecal and urinary magnesium. At a low intake of calcium, PTE animals showed lower plasma and higher faecal magnesium than intact animals. In intact animals, urinary magnesium was higher on the low level of dietary calcium than on the normal intake of calcium.

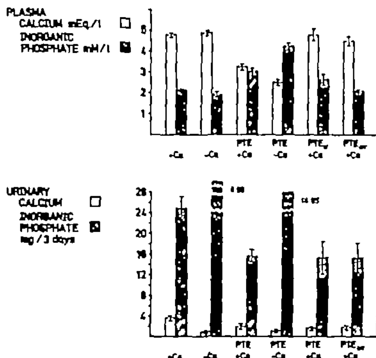


Fig 6 Plasma and urinary levels of calcium and inorganic phosphate. The contents of inorganic phosphate in plasma showed an inverse relationship to those of calcium. Note the highly increased urinary phosphate in the calcium-deficient animals compared to normal. On a normal level of dietary calcium, PTE resulted in reduced urinary phosphate. The high contents of urinary phosphate found in the calcium-deficient PTE animals was certainly due to an increased clearance of the markedly elevated plasma phosphate in these animals.

Plasma and Urinary Calcium and Inorganic Phosphate

In plasma the contents of inorganic phosphate showed an inverse relationship to those of calcium (Fig 6) The calcium-deficient intact animals showed somewhat lower values for inorganic phosphate than those of the normal controls, the difference being almost significant. At a normal intake of calcium, the PTE animals showed significantly higher values than those of the intact animals. At a low intake of calcium, the phosphate values were highly increased in the PTE animals compared with those of the intact animals. The group of PTE animals fed the low calcium diet showed the highest values for inorganic phosphate and the lowest values for plasma calcium in the whole series. In the remaining two groups of rats, no significant differences from normal were found. Urinary phosphate was increased in calcium-deficient intact and PTE animals and reduced in the remaining experimental groups (Fig 6)

DISCUSSION

Plasma calcium in the intact animals showed no significant difference between rats fed the low calcium diet and those supplied the calcium supplemented diet, while the PTE animals showed plasma calcium which was highly dependent on the level of dietary calcium. The criterion of an absence of parathyroid function and the efficiency of parathyroidectomy has been discussed in a previous study (*Larsson* & *Ahlgren* 1975) At the low level of dietary calcium, the striking difference between the intact and the PTE animals is best explained by increased secretion of parathyroid hormone when the intake of calcium is low as demonstrated by the occurrence of parathyroid hyperplasia (*Sevastikoglou* & *Larsson* 1972) In the present study this is also indicated by the highly increased urinary inorganic phosphate and reduced urinary calcium in the calcium-deficient intact animals compared with that of the calcium supplemented normal controls. At a normal intake of calcium, PTE resulted in a reduced urinary excretion of inorganic phosphate and increased contents of phos-

phate in plasma. This was also found at the low level of dietary calcium. Although urinary phosphate was lower in the calcium-deficient PTE animals than in the calcium-deficient intact animals, it was higher than that in the intact control animals fed the calcium supplemented diet. This difference was certainly due to an increased clearance of inorganic phosphate by the kidneys of the calcium-deficient PTE animals which had markedly elevated plasma values compared to those of the normal controls (*Thompson* & *Hiatt* 1957). This finding confirms that recently reported by *Clark* & *Rivera-Cordero* (1973) who observed the phenomenon in rats. It has also been reported to occur in man (*Eisenberg* 1966).

The group of calcium supplemented PTE animals showing normalized plasma calcium as well as the group of animals with a non-verified PTE showed reduced urinary inorganic phosphate corresponding to that found in the group of calcium supplemented rats with a verified PTE showing reduced plasma calcium. These findings may suggest a state of hypoparathyroidism. However urinary calcium was also reduced in these animals in which plasma contents of calcium and inorganic phosphate were within normal limits. This is not directly in line with a condition of hypoparathyroidism, solely. In two animals in the group in which the effect of PTE could not be verified, one of the parathyroids was found to have been left intact (*Larsson* & *Ahlgren* 1975). The findings are best explained by an increased activity of metabolites from vitamin D which is thought to increase the reabsorption of urinary inorganic phosphate (*Borle* 1963) and calcium (*Gran* 1960) thus promoting the retention of both phosphate and calcium. Further studies are needed, however, to elucidate the effects of vitamin D on the kidneys.

As demonstrated by *Nicolaysen et al.* (1953) vitamin D is necessary for the adaptation to a low calcium intake. The regulation of the production of active metabolites of vitamin D is not well known. The metabolite 1,25-dihydroxycholecalciferol, active in intestine (*Holick et al.* 1971, *Myrtle* & *Norman* 1971) and bone (*Raisz et al.* 1972, *Reynolds et al.* 1973) shows lower concentrations in parathyroidectomized chicks than in rachitic controls, both in plasma and in intestine, and it has been suggested that parathyroid hormone is the major regulator of the production of this metabolite (*Garabedian et al.* 1972, *Fraser* & *Kodicek* 1973). This has also been suggested from the finding that parathyroid extract suppresses the production of 1,25-dihydroxycholecalciferol in the rat (*Galante et al.* 1972). However the results of the present investigation are contradictory to this theory since there was no impairment of the intestinal net absorption of calcium in the PTE animals either at a normal or at a low level of calcium in the diet. Thus, the calcium-deficient PTE animals showed no disturbance of the ability to increase the intestinal absorption of calcium adaptory to the reduced intake of dietary calcium. This was clearly demonstrated by the increased levels of the plasma ^{47}Ca-activity recorded after administration of the isotope by stomach tube and also by the reduced faecal ^{47}Ca-activity. The calcium-deficient PTE animals showed in fact, higher plasma ^{47}Ca-activity, lower faecal ^{47}Ca activity and twice as high intestinal net absorption of calcium than the calcium-deficient intact animals.

In spite of a reduced calcium accretion rate by bone in the PTE animals both at a normal and a low intake of dietary calcium (*Larsson* & *Ahlgren* 1975) there was an increased retention of ^{47}Ca by bone due to the increased plasma ^{47}Ca level caused by the increased intestinal ^{47}Ca absorption. In the calcium-deficient intact animals, the increased retention of ^{47}Ca by bone was caused by increased calcium accretion rate (*Larsson* & *Ahlgren* 1975) as well as by increased plasma ^{47}Ca level. This was also found in the PTE animals showing a normalized plasma calcium level.

While the excretion of urinary calcium at a normal intake of dietary calcium was reduced in the PTE animals, the urinary ^{47}Ca activity showed no significant change

due to the increased plasma ^{45}Ca specific activity. At a low calcium intake the excretion of urinary calcium was reduced in the intact animals, certainly due to increased tubular reabsorption of calcium caused by increased parathyroid activity (*Kleeman et al.* 1961) while urinary calcium in the PTE animals was reduced because of the low plasma calcium level. Thus, in the intact animals, the urinary ^{45}Ca activity was reduced while it in the PTE animals was increased due to the increased plasma ^{45}Ca specific activity.

The contents of inorganic phosphate in plasma showed an inverse relationship to those of calcium. While vitamin D appears to be the most important factor influencing phosphate absorption (*Harrison & Harrison* 1961) the role of parathyroid hormone is not quite clear. It has been reported that parathyroid hormone does not cause any significant changes either in balance or in net absorption of phosphate (*Cramer et al.* 1961) and, similarly it has been found that parathyroidectomy does not affect the intestinal phosphate absorption (*Wasserman & Comar* 1961). However experiments on perfused intestinal loops *in vitro* have shown an increased transport of phosphate from mucosa to serosa caused by parathyroid hormone (*Borle et al.* 1963). Recently calcitonin has been reported to prevent the rise in plasma phosphate concentration which otherwise would occur during infusion or following oral administration of calcium (*Talmage et al.* 1973). In the present investigation the variations of the plasma concentrations of inorganic phosphate appear to be related to the induced variations of the endogenous secretion of parathyroid hormone and its effect on urinary phosphate excretion.

As to magnesium, increased faecal and reduced plasma concentrations were found in the group of calcium-deficient PTE animals indicating a reduced absorption of magnesium in the intestine in agreement with the report by *Jones & [illegible]rman* (1966). The increased urinary m[illegible]sium recorded in the group of calcium-def[illegible]nt intact animals was probably caused by the increased parathyroid activity. The excretion of magnesium falls after parathyroid hormone injection but later increases as magnesium is mobilized from bone (*Gill et al.* 1967).

In overall conclusion, a marked intestinal adaptation viz. a distinctly increased efficiency of calcium absorption, occurred in the calcium-deficient PTE rats indicating that the intestinal calcium absorption which is regulated by the action of metabolites of vitamin D is not disturbed. Thus, the metabolism of vitamin D does not appear to be regulated by parathyroid hormone, but probably directly by changes in the plasma calcium level. Studies are in progress to elucidate the metabolism of vitamin D at varying plasma calcium levels in PTE animals. Urinary calcium excretion was reduced because of the reduced plasma calcium level. As shown in a previous study (*Larsson & Ahlgren* 1975) selective PTE resulted in a reduced ability to mobilize skeletal calcium necessary for an adaptation to a low calcium intake. While intact animals developed calcium-deficiency osteoporosis, PTE animals did not.

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7,12 DMBA-INDUCED RAT MAMMARY TUMOUR STUDIED FOR HORMONAL RESPONSIVENESS *IN VITRO*

I Short-term Incubations of Cell Suspensions

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Aspegren, K. 7 12-DMBA-induced rat mammary tumour studied for hormonal responsiveness *in vitro*. I Short-term incubations of cell suspensions. Acta path. microbiol. scand. Sect. A 83 25–36, 1975

The effect of testosterone, progesterone, and 17-β-oestradiol on the incorporation of tritiated thymidine into DNA was studied *in vitro* using cell suspensions from DMBA-induced rat mammary carcinomas and subcutaneous sarcomas. Both stimulatory and inhibitory effects of the hormones were seen. The two types of tumour reacted similarly. The validity of hormonal responsiveness *in vitro* compared with *in vivo* is discussed.

Key words: Mammary tumour rat hormonal responsiveness cell suspension.

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A mammary carcinoma can be induced in the 50-day-old female Sprague Dawley rat by the intragastric administration of 20 mg 7 12-dimethyl(a)benzantracene (DMBA) Oophorectomy prevents the tumour induction and often causes regression of established tumours which are then called hormone sensitive.

The reactivity of this tumour to steroid hormones has been investigated by various workers and the results can be summarized as follows. A testosterone propionate dose, 50 mg/kg/day given intramuscularly to intact animals does not influence tumour growth, whereas 3 or 6 mg/kg/day represses the tumours in more than 80 per cent of the rats (4) Progesterone administered as intraperitoneally implanted pellets (giving a dose of about 3 mg/rat/day) does not influence tumour growth in intact animals (6) Oestradiol given to intact animals in doses of 20 µg/rat/day delays the appearance of the tumours and depresses growth of established tumours, but does not extinguish the latter (5) Using doses of 6.25 mg/kg/day *Teller et al.* (16) induced complete tumour remission in 67 per cent of the rats. Oophorectomy does not induce regression of some tumours and 37.5 per cent of these failures can be converted to complete remissions by large doses of oestradiol.

Prolactin seems to play a major role for the growth of the tumour *Nagasawa & Yanai* (12) studied its effect in adreno-oophorectomized animals and found growth stimulation at 5 mg/rat/day *Sterental et al.* (15) induced reactivation of growth in adreno-oophorectomized animals, but failed to do so if hypophysectomy was performed. *Butler &*

Pearson (2) found regression of tumour growth in animals treated with rabbit antibodies to purified rat prolactin.

Numerous *in vitro* investigations of mouse and rat mammary tumours are available, but only few investigators use the 7 12 DMBA induced tumour *Welsch & Rivera* (21) studied the tumour in organ culture and could demonstrate stimulatory effects of prolactin, but using 5 or 10 μg/ml of oestradiol nothing but inhibitory effects were seen The normal, non-neoplastic mammary gland of the Sprague-Dawley rat was studied in organ culture by *Koyama et al* (10) who found no definite effect of oestradiol in the concentration range of 0 001–1 0 μg/ml. Using cortisol, they found an inhibitory effect at 5 0 μg/ml and using progesterone a stimulatory effect was obtained by 0.1 and 1 0 μg/ml.

In a previous publication (1) the direct effect of androsterone, progesterone and oestradiol at various concentrations was studied *in vitro* in short term incubations of cell suspensions prepared from human mammary carcinoma. As the 7 12 DMBA rat mammary tumour shows many of the endocrine characteristics of human mammary carcinoma, it has been claimed as a model tumour for studies of human mammary carcinoma. It was therefore judged to be of interest to perform a similar *in vitro* study of the 7 12 DMBA tumour to ascertain similarities or dissimilarities between the two species.

MATERIAL AND METHODS

The mammary tumours were induced in 50-day-old outbred female Sprague-Dawley rats (Anticimex, Stockholm) by intragastric instillation of 20 mg 7 12 DMBA in oil suspension. The animals were used within six months, reckoned from the day of induction. Histologically all tumours were adenocarcinomas. *In order to obtain control tumours*, sarcomas were induced in 50-day-old rats by subcutaneous injection in the back of either 0.4 mg of methylcholanthrene or 2 mg of 7 12 DMBA. All were histologically verified sarcomas Three virus-induced rat sarcomas were also studied one Rous sarcoma, one polyoma sarcoma, and one adeno-12 sarcoma. Each of the virus-induced sarcomas had been transplanted for at least 12 generations before the *in vitro* studies.

The cell suspension culture technique is described in detail by *Nordqvist* (13) and by *Aspegren & Håkansson* (1)

Briefly a cell suspension is obtained by mincing a tumour with scissors in Parker 199 medium (SBL, Stockholm) and filtering the suspension through double gauze. The cells are suspended in Parker 199 with pooled 20 per cent human heat inactivated serum added. Heparin 8,000 IU/ml (Vitrum, Stockholm) at a concentration of 0.016 IU/ml and sodium benzylpenicillin and streptomycin sulphate at final concentrations of 50 IU/ml and 50 μg/ml respectively are added. The cells are distributed in tubes, each containing 1 ml medium, and are incubated at 37 C during continuous stirring.

The incubation is terminated by spinning down the cells and washing these with cold phosphate buffer The cells are then extracted for 30 minutes with cold 5 per cent trichloroacetic acid (TCA) washed once with cold TCA, and once with absolute ethanol. The residue is stored at –20 C until further processed.

Radioactivity and DNA determination The frozen incubates were thawed and dissolved in 1 ml of 1 N NaOH at 37 C for 1 hour 0 1 ml of this solution was dissolved in 1 ml Soluene 100 (Packard) and 14 ml scintillation fluid (300 mg dimethyl-POPOP and 5 g PPO in 1,000 ml toluene) was added. The radioactivity of the sample was assayed in a Packard Tri-Carb 3310 scintillation counter during 10 minutes counting. Efficiency was continuously checked with external standardisation. DNA was determined on two 0.2 ml portions of the sample by means of a modified indole method. An adjusted DNA value was obtained as described earlier (1) by adjustment against a standard curve The cell suspensions were studied in two different experimental models.

Model I

The cells were distributed in tubes containing 1 ml of medium with 1 per cent ethanol (controls) The tubes were incubated at 37 C during continuous stirring. During the last hour of incubation, either 2 μC H^3-thymidine or 2 μC 5-H^3-uridine (Schwartz Mann, Orangeburg, NY) was added. Three tubes were set up for each determination. Incubation lasted for 1 2, 3 4 or 5 hours and was terminated as described above. DNA content and isotope incorporation were determined from these incubations, and DNA values were obtained also from three similar tubes terminated immediately after setting up the cultures (time 0) Two mammary carcinomas and two sarcomas were studied in this way

Model II

The cells were distributed in tubes, each containing 1 ml of medium and incubated at 37° C during continuous stirring. Four tubes were set up as controls with only ethanol added and two tubes for each hormone were tested. The hormones were obtained from Sigma (St. Louis, Mo.) and were dissolved in 96 per cent ethanol to give a final ethanol concentration in the medium of 1 per cent. Control cultures contained 1 per cent ethanol only. In one series of incubations, the following hormones were used at the stated final concentrations

Testosterone 0.5, 5, and 50 μg/ml

Progesterone 0.8, 8, and 80 μg/ml

17-β-oestradiol: 0.02, 0.2, 2, and 20 μg/ml.

In another series, testosterone, progesterone, 17-α-oestradiol, 17-β-oestradiol, and cholesterol were used at 20, 50 and 80 μg/ml, respectively.

Incubation time was 3 hours, during the last hour 2 μC H³-TdR was added and the cultures were treated as described above.

Statistical treatment

As demonstrated by *Nordqvist* (13) the cpm/DNA ratios are not normally distributed in the cell suspension system, but a near normal distribution can be obtained through logarithmation. Thus cpm was related to DNA as follows

$$\log_e \left\{ \frac{\text{cpm} \times 10^4}{(\text{Ext. std.}) \times (\text{DNA})} \right\}$$

These figures were multiplied by 100 for convenience of handling and were called *a*-values.

The determinations from identically prepared cultures were used to assess the technical error variance, uniformity being tested by Bartlett's test. Ten out of 486 double or quadruple determinations were found to deviate significantly from the others and were excluded from the estimate of general error variance and the analysis of interaction. The possible effects of the addition of hormones on DNA synthesis during incubation were evaluated as the difference between the *a*-values of hormone-treated and control cell suspensions. The statistical significance of such difference was judged by analysis of variance as described previously (1).

The reaction of various tumours was tested by an analysis of interaction between the two sources of variation "between tumours" and "between control and hormone-treated tubes".

RESULTS

Technical Considerations

Fig. 1 shows isotope incorporation and DNA content of cell suspensions prepared from two mammary carcinomas (Fig. 1 A) and two sarcomas (Fig. 1 B) studied for five hours after the start of the incubation which occurred in the absence of added hormones. Thymidine and uridine incorporation decline only slightly whether studied as direct counts or as *a*-values (Fig. 1 C-D). The DNA content of the suspensions declines more markedly and after 5 hours of incubation only 60–70 per cent of the initial DNA amount remains. The biggest changes in DNA content apparently occur during the first one or two hours of incubation. Thereafter the system is relatively stable for at least five hours.

The effect of added hormones on thymidine incorporation was studied in two different sets of experiments. In the first, sixteen mammary carcinomas and fifteen sarcomas with different hormone concentrations were studied. Fig. 2 A-C shows the registered differences between *a*-values in the presence of a certain hormone and the corresponding control *a*-value (with only ethanol added). The significance levels marked in this figure are based on a comparison with the general technical error variance and thus mark the levels where it is unlikely that the noted effects are due to random variations caused by the technical errors involved in the method. As many comparisons have been made single effects might by chance reach the * and even the ** levels.

To study the effect variations caused by the experimental conditions, a cell suspension from a mammary tumour was prepared and distributed to glass tubes in groups comprising three control tubes and three hormone tubes. 17 β-oestradiol at 50 μg/ml was used for this experiment. Five such groups were set up. For each group different media and different pipettes were used, and the ethanol for the control tubes was drawn from five different sources, whereas the hormonal solution was the same for all groups. Thus the possible sources of variations caused by experimental variables could be studied. The recorded values were tested for interaction between the two sources of variation, "between effects" and "between experimental variations"

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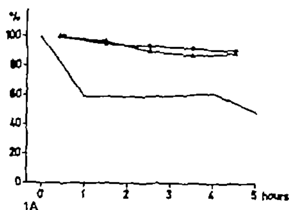
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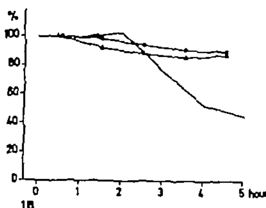
Technical Considerations

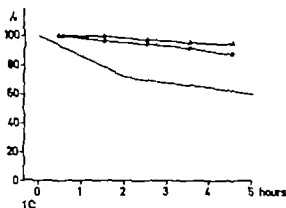
Fig. 1 shows isotope incorporation and DNA content of cell suspensions prepared from two mammary carcinomas (Fig. 1 A) and two sarcomas (Fig. 1 B) studied for five hours after the start of the incubation which occurred in the absence of added hormones. Thymidine and uridine incorporation decline only slightly whether studied as direct counts or as *a*-values (Fig. 1 C-D). The DNA content of the suspensions declines more markedly and after 5 hours of incubation, only 60–70 per cent of the initial DNA amount remains. The biggest changes in DNA content apparently occur during the first one or two hours of incubation. Thereafter, the system is relatively stable for at least five hours.

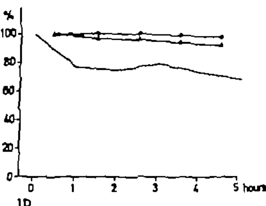
The effect of added hormones on thymidine incorporation was studied in two different sets of experiments. In the first, sixteen mammary carcinomas and fifteen sarcomas with different hormone concentrations were studied. Fig. 2 A-C shows the registered differences between *a*-values in the presence of a certain hormone and the corresponding control *a*-value (with only ethanol added). The significance levels marked in this figure are based on a comparison with the general technical error variance and thus mark the *levels where it is unlikely that the noted effects are due to random variations caused by the technical errors involved in the method.* As many comparisons have been made, single effects might by chance reach the * and even the ** levels.

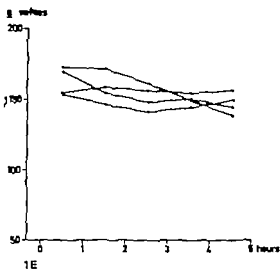
To study the effect variations caused by the experimental conditions, a cell suspension from a mammary tumour was prepared and distributed to glass tubes in groups comprising three control tubes and three hormone tubes. 17 β-oestradiol at 50 µg/ml was used for this experiment. Five such groups were set up. For each group different media and different pipettes were used, and the ethanol for the control tubes was drawn from five different sources, whereas the hormonal solution was the same for all groups. Thus the possible sources of variations caused by experimental variables could be studied. The recorded values were tested for interaction between the two sources of variation, "between effects" and "between experimental variations"











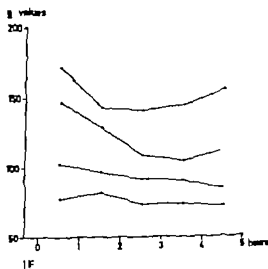
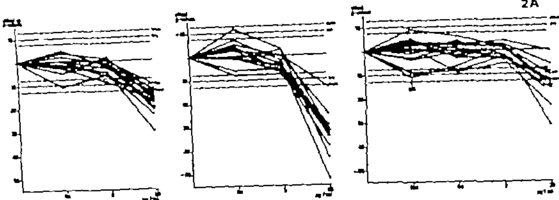
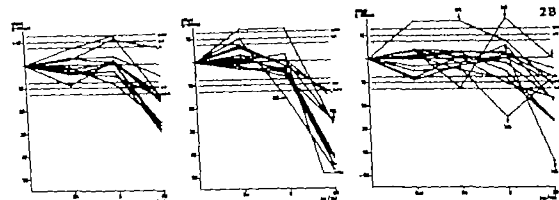


Fig 1 Changes in incorporation of tritiated thymidine (-●-●-) and tritiated uridine (-Δ Δ-) and in the DNA content (—— —) during five hours incubation of two DMBA-induced rat mammary tumours (Fig 1 A and 1 B) and two DMBA-induced subcutaneous rat sarcomas (Fig 1 C and 1 D). The incorporation is expressed as percentage of th log c.p.m. during the first hour The DNA content at each hour is expressed as percentage of the DNA content of the freshly prepared cell suspension. Fig. 1 E and 1 F show the corresponding s-values applying to tritiated thymidine and uridine respectively





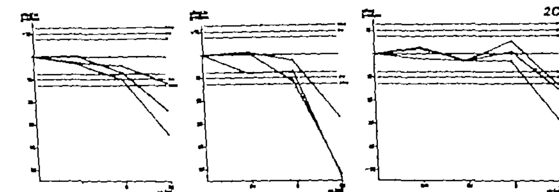


Fig. 2 A. Effects of testosterone, progesterone, and oestradiol on the incorporation of H^3-TdR into sixteen rat mammary carcinomas. The effects are expressed as differences in $\log_{10}$ c.p.m./DNA ratios (a-values) in controls and hormone-treated samples. Effects above the zero line represent stimulation; below the line, depressions of incorporation. The determinations from a single tumour are joined by solid lines to represent effect curves. Arrows represent tumours with deviating error variances.

Fig. 2 B. Effects of testosterone, progesterone, and oestradiol on the incorporation of H^3 TdR into cells from twelve primarily-induced rat sarcomas. For explanations, see text to Fig. A.

Fig. 2 C. Effects of testosterone, progesterone, and oestradiol on the incorporation of H^3 TdR into cells from three virus-induced and serially transplanted rat sarcomas. For explanations, see text to *Fig. 2 A.*

but none was found (F = 1.03 NS at 4 and 20 df.)

Variations caused by "animals" and "experiments" were indirectly studied in the six incubations that used cholesterol which did not affect thymidine incorporation (see below). When these were tested for interaction between "tumours" and "effect" none was found. Here the heading "tumour" includes not only the source of variation "tumour" *per se* but also "experiments", "animals" and "experimental days" which thus appear to be insignificant.

Testosterone

The general picture to be seen if testosterone is added to the medium is that of an inhibition of thymidine incorporation at increasing concentrations, but a few tumours deviate from the others in their response. In order to evaluate whether such deviations can occur by chance, an analysis of variance was performed to study the interaction between the two sources of variation "between tumours" and "between control and hormone". Table 1 shows the results.

At 0.5 μg/ml, there is no evidence to suggest that different tumours react differently. It is true (Fig. 2 A) that one mammary carcinoma appears to be more strongly inhibited than the other tumours and even reaches the ** level. As a total of 31 tumours were studied, this phenomenon might be random, an explanation supported by the observation that the same tumour suspension showed a less marked inhibition of thymidine incorporation at 5 μg/ml of testosterone (Fig. 2A). Most likely all tumours react in a similar way showing a mean inhibition of only 1.2 e units with an error of 0.64. Thus, this inhibition is not statistically significant, but might be true as increasing hormone concentrations result in further inhibition.

At 5 μg/ml, a significant interaction is seen to apply to the sarcoma group (Table 1)—thus the behaviour of one or more sarcomas may differ from that of the others. A marked stimulation is seen in two 7,12 DMBA induced sarcomas, amounting to effects of +10.9 (No. 2) and +12.6 (No. 1) e units. If these two are excluded, no residual interaction is found (F = 1.43 NS at 12 and 476 df.). The remaining tumours (mammary and sarcomas) show a mean inhibition of -2.52 ± 0.69 ($t = 3.7$, $P < 0.001$ at 27 df.) which thus is weak although statistically significant. Compared with this mean, both deviating sarcomas show marked stimulations ($t = 3.9$ and $t = 3.5$ both at 476 df., $P < 0.001$). Thus, the behaviour of these two tumours cannot be explained by random variations from the technical errors.

At the highest testosterone concentration, a marked inhibition is seen and the variability in response of different sarcomas is highly significant (Table 1). The main inhibitory effect of cells from mammary carcinoma and cells from sarcomas is the same, but the latter scatter more around this mean and one sarcoma (No. 2) shows a marked deviation as it is stimulated at this high testosterone concentration. The elimination of that sarcoma from the calculation still leaves a residual heterogeneity apparent as a strongly significant interaction (F = 7.19, $P < 0.001$ at 13 and 476 df.). The mean inhibition of all other tumours is -18.1 ± 3.11 units which obviously is highly significant, ($t = 5.8$, $P < 0.001$ at 19 df.).

The stimulated sarcoma is the same as one of the two that were stimulated also by 5 μg/ml of testosterone.

Progesterone

The pattern to be seen if progesterone is used (Fig. 2) resembles that described for testosterone. As shown in Table 1, different tumours might react differently both at 0.8 and 8 μg/ml—this phenomenon disappears when one mammary carcinoma and one sarcoma (No. 2) are excluded from the test of interaction performed for values at 0.8 μg/ml (F = 1.18, NS at 28 and 476 df.). Both tumours show stimulations that reach statistical significance, but at least one of these could well be random. Sarcoma No. 2 is markedly

TABLE 1 *Analysis of Variance in a Study of the Variability among Tumours with Respect to Response to Hormones at Different Concentrations*

Hormone	Concentration μg/ml	Mammary tumours df.	Variance	F	Sarcomas df	Variance	F	Total material df.	Variance	F
Testosterone	0 5	15	18 53	<1	14	22.94	1 14NS	30	20.72	1.03NS
	5	14	12.15	<1	14	53.63	2.67***	29	35.61	1 77**
	50	15	31 75	1.58NS	14	203.59	10.12***	30	118.04	5.87***
rogesterone	0.8	15	34 15	1 70*	14	32.15	1.60NS	30	34.61	1 72*
	8	15	15.25	<1	13	51.90	2.58**	29	32.69	1.62
	80	15	88.83	4 42***	12	310.91	15 45***	28	182.79	9.09***
7-β-oestradiol	0 02	14	23.83	1 19NS	14	42.25	2.10*	29	32.13	1.60*
	0.2	15	25.54	1.27NS	13	26.68	1.33NS	29	25.24	1.25NS
	2	15	10 70	<1	12	39 10	1.94	28	22.58	1 12NS
	20	15	64.30	3.20***	11	106.80	5.31***	27	78.07	3.88***

Variances and degrees of freedom (df.) due to interaction between two sources of variation are given: "between tumours" and "between controls and hormone treated cell suspensions" Variance ratios (F-values) compare these variances with the general technical error variance (20.12 based on 476 df.)
* 0.05 > P > 0.01 ** 0.01 > P > 0.001 *** P < 0.001

stimulated also at 8 μg/ml and then causes a significant heterogeneity between the tumour responses (Table 1) After exclusion of the latter sarcoma, no residual interaction remains (F < 1) the main effect being that of an inhibition, –3.6 ± 0 65 *a* units (t = 4.64 at 27 df., P < 0.001)

At 80 μg/ml, there is a general, strong inhibition both of mammary carcinomas and sarcomas with a mean effect of –34 6 ± 2.07 *a* units, no marked deviations being observable in any specific tumour Once more, the scatter of the values denoting effect is more marked than that otherwise to be expected from random effects of the technical error

Oestradiol

As regards oestradiol (Fig. 2) an interaction between the sarcomas is probably present at the lowest concentration, 0.02 μg/ml, due to a stimulation noted in one sarcoma. If the latter is excluded, the remainders show a slight and non-significant stimulation with a mean effect of +0.54 ± 0.80 *a* units. The stimulation noted in the single sarcoma (No. 2) is significant (t = 3.53 0.01 > P > 0.001 at 29 df) The same sarcoma shows a high value also at 0.2 μg/ml, but the registered technical error variance between the two double determinations is unusually large, which is the reason why this value was excluded from the analysis of variance. The noted stimulation can be a result of a technical mishap it can also be an actual stimulation. The result obtained at the lower concentration supports the latter interpretation. Among the other tumours, one sarcoma deviates in that arrest is more marked than that of the others. But this deviation can be a random variation around the noted mean effect, +1 16 ± 0 78 *a* units, as no significant interaction is found (Table 1)

At 2 μg/ml of oestradiol, a probable interaction exists in the sarcoma group this is due to one sarcoma (No. 1) which is somewhat more inhibited than the others. It is possible, however that it represents a random variation of the mean effect value +0 07 ± 0 70

Thus, at levels up to 2 μg/ml, there is no indication of a general inhibiting effect of oestradiol, but a few tumours deviate in their response. Among these the stimulation seen in the case of sarcoma No 2 is probably not random, but others could well be. At the highest concentration of oestradiol, 20 μg/ml,

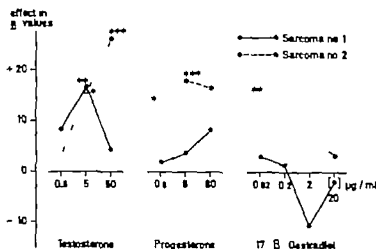


Fig 3 Effects of hormones on sarcomas No. 1 and 2 compared with mean effects of other sarcomas. For explanations, see text.
** denotes $0.01 > P > 0.001$
*** $P < 0.001$

interaction is strong and highly significant, indicating a large variability in response one sarcoma and one mammary carcinoma are virtually non-inhibited while the remainders scatter some showing a very strong inhibition. The mean inhibition of all tumours studied at this concentration amounts to -14.9 ± 1.37 ε units. If the non-responding tumour is excluded from the mammary group there is still a highly significant interaction between the remaining tumours ($F = 2.25$ $0.005 > P > 0.001$ at 14 and 476 df)

Nine effect values had to be excluded from the analysis so far performed because of an increased variance in technical errors at these determinations. These values are specially marked in Fig 2 A-C. None of these show a statistically significant difference from the mean effect at the corresponding concentration if tested by t tests.

Obviously most tumours react in a similar way to the hormones with a dose-dependent inhibition of thymidine incorporation only two tumours out of 31 studied have shown responses that deviate so much that they cannot be random.

Both are 7 12 DMBA induced sarcomas (No. 1 and 2) Fig 3 plots the differences between the recorded effects and the average tumour effect at different hormone concentrations in these two tumours. It could be argued that the recorded "stimulations" were due to technical mishaps affecting the control groups, resulting in an underestimation of the control thymidine incorporation. Four parallel tubes were always studied in the control groups, but theoretically a slight contamination by some toxic factor could occur e.g., by the use of a contaminated pipette for the ethanol solution. Against this explanation argues that stimulations as well as inhibitions are recorded in the case of No. 1 and some effect values are quite similar to the expected values. Reduced control values should result in increases of all effect values, irrespective of hormone and concentration used.

As regards No. 2, however only stimulations are recorded, which is in contrast to the response of the average tumour (see Fig. 3) If these are due to an underestimation of the control thymidine uptake, then the stimulations above the average tumour responses should not vary to degrees higher than those accounted for by technical variations, as long as the highest concentrations are not included in the analysis. In the case of this tumour six such effect values are available (testosterone 0.5 and 5 μg/ml, progesterone 0.8 and 8 μg/ml, oestradiol 0 02 and 2 μg/ml Oestradiol 0.2 μg/ml cannot be included in the analysis, the technical error variance being too large,

[1] The error of the differences between means in control group and hormone-treated group and the number of df's were calculated, as described by *Welch* (20)

as stressed above) The mean of these six values is 11 74 and their variance is 31.86. As each effect value is based on two determinations based on data involving a general technical error variance of 20.12, the values should have a variance of $20\ 12 \times \left(\frac{1}{2}+\frac{1}{28}\right)=10.78.$

Thus, the actual variance found exceeds the expected variance, and a variance ratio gives $F = 2.96$ at 5 and 476 df., $0\ 02 > P > 0\ 01$ Accordingly it is hardly likely that a general depression of the four control values should be the explanation of the deviation of sarcoma No 2. This is further supported by the fact that the effects shown in Fig 3 apparently form dose-related graphs and do not appear haphazardly

To clarify "unspecific toxicity" versus "specific effect" of the steroids in the present system, incubations of six different mammary tumours were performed at concentrations of 20 50 and 80 μg/ml of all five steroids. All six tumours were incubated with all concentrations of 17-α-oestradiol and cholesterol three of the tumours were incubated with 17 β-oestradiol at 20 μg/ml, testosterone at 50 μg/ml, and progesterone at 80 μg/ml only the remaining three were incubated with all concentrations of the latter hormones. Fig 4 shows the results.

The significance of the hormonal effects was tested by t tests Table 2 gives the results. Cholesterol has no effect on the incorporation of H^3 TdR into DNA, whereas 17 β-oestradiol is a strong inhibitor Progesterone and testosterone give about half the effect of 17 β-oestradiol, whereas 17-α-oestradiol has a smaller but significant effect.

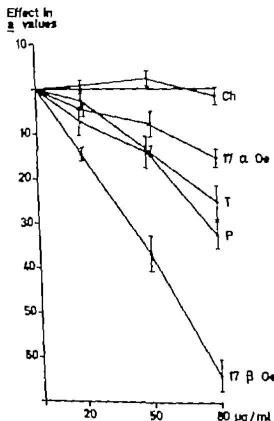


Fig 4 Comparison of the effects of cholesterol, testosterone, progesterone, 17-α-oestradiol, and 17 β-oestradiol on six different DMBA-induced rat mammary tumours. Each point represents the mean of three or six determinations and is given with its standard error Table 2 gives the significance of the deviation of each point from the zero line.

DISCUSSION

Short-term incubation of cell suspensions was used to study the reaction of tumour cells to hormones *in vitro* During the two hours incubation period before the addition of tritiated thymidine a reduction in cell number obviously occurs, as the DNA content of the culture is reduced to appr two-thirds of its initial value. There is also a moderate decrease in DNA and RNA synthesis, as studied by nucleoside incorporation, but the mode of estimation used—that in which the so-called a values logarithmically expresses isotope incorporation per DNA amount—implies that the reduction during every five hours of incubation amounts to only appr 10 per cent. Part of the explanation of the initial cell death could be direct cell injury during the preparation of the cell suspensions, but it is also possible that further cell death is attributable to the incubation system used. It cannot be decided whether cell death is a random phenomenon or whether certain cell

TABLE 2. *Significance of Dose Response Curves of Different Hormones as Given in Fig 4*

Concentration µg/ml		d.f.	t	P
Testosterone	20	2	1.6574	NS
	50	5	11.5519	**
	80	2	6.4998	*
Progesterone	20	2	2.4063	NS
	50	2	3.9745	NS
	80	5	10.5918	**
17-β-oestradiol	20	5	10.0083	***
	50	2	9.2113	*
	80	2	17.2863	***
17-α-oestradiol	20	5	2.7067	NS
	50	5	2.8541	
	80	5	7.0894	***
Cholesterol	20	5	0.6982	NS
	50	5	1.2612	NS
	80	5	0.9516	NS

Student's t-test was performed on the effect values. t-values with degrees of freedom are given.
$0.05 > P > 0.001$ ** $0.01 > P > 0.001$ ** $P < 0.001$

types or clones survive better than others. Possibly a selection occurs in the cell material but if so, it should reasonable be less pronounced than otherwise seen when actual tissue culturing is performed, either as monolayer culture or as organ culture.

The problem of "toxicity" of steroid hormones is studied in the experiments utilizing cholesterol and the α isomer of oestradiol. Cholesterol does not bind to oestrogen receptors of target cells (8) but it does pass the cell membrane freely (14). In the present investigation, it had no inhibitory or stimulatory effect on mammary tumour cells in six different experiments. 17-α-oestradiol binds to oestrogen receptors of DMBA mammary tumour in a way similar to 17 β-oestradiol, although to a lesser extent (18). *Korenman* (9) using cytosol from rabbit uteri, found that the binding of 17-α-oestradiol was 49 per cent of that of 17 β-oestradiol. *Teller et al.* (16) compared the potency of 17 β-oestradiol and 17-α-oestradiol to induce regression of DMBA induced rat mammary tumour and found that the α isomer gave significantly less remissions. This agrees with the present investigation.

The inhibitory effects of the steroids in the concentration range of 20–80 µg/ml in cases of rat mammary tumours and rat sarcomas cannot reasonably be merely "toxicity" due to the presence of a large concentration of steroid molecule, but rather that each hormone has a "specific toxicity" as stated by *Nordqvist* (13).

The present study questions whether hormonal responsiveness *in vitro* mirrors actual hormonal responsiveness *in vivo* of DMBA induced mammary tumour in the rat.

As it can be argued that primarily induced subcutaneous rat sarcomas are unsuitable for studies of hormonal responsiveness because of unpredictable derepression of the genome three different virus-induced and serially transplanted sarcomas were also used. In all three inhibitions at high steroid concentrations were seen. The *in vitro* method used here was previously by *Nordqvist* (13) applied to human endometrial cells, normal and malignant, and by *Aspegren & Håkansson* (1) to human mammary carcinoma cells. In both studies, marked thymidine incorporation inhibition resembling that seen here was observed. *Nordqvist* however also investigated

three human gastro-intestinal carcinomas, and *Aspegren & Håkansson* studied two human sarcomas any effects were not noted in any of these five tumours, even at the highest concentrations of hormones. The discrepancy of the results obtained from the rat tumours used here might be due to species differences, but could also indicate that the "toxicity" is less unspecific than might at first appear

In the present study a few tumours deviate from the general pattern. Some of these deviations might be random variations or could be due to technical errors. In two tumours (discussed at some length above) however marked deviations occurred which could not be explained. Both were sarcomas. In one, thymidine incorporation was markedly increased at certain concentrations of all three hormones in the other stimulation by testosterone and inhibition by oestradiol was seen. These hormonal effects are reasonably not caused by technical errors or experimental mishaps.

Hormonal responsiveness *in vivo* of primarily-induced subcutaneous rat sarcomas was studied by *Mizukami et al.* (11) They induced sarcomas in male Wistar rats by DMBA and studied whether castration alone or castration and stilboestrol treatment had any effect, and found no effect on tumour incidence and a possible effect on growth. These treatments are both known to influence incidence (5) and growth (3) of the 7 12 DMBA rat mammary tumour The rat sarcoma should therefore at least be regarded as less hormonal responsive than the rat mammary carcinoma. None the less, under the *in vitro* conditions used in the present study sarcoma cells could be influenced by the steroids. In most instances, only the inhibiting effect to set in at high concentrations was noted, but in two sarcomas, other effects of the hormones, partly consisting of *stimulations* of isotope incorporation, were seen at lower concentrations.

The mode of action of the steroid hormones on DNA synthesis in the present system is unclear Specific control of gene expression has been much discussed (7) but other modes of reaction to steroids might exist, i.e. "pleiotypic responses" as suggested by *Tomkins* (19) Possibly the artificial conditions of *in vitro* testing in the present study somehow enable cells to react. Another *in vitro* technique has therefore been used, and the results will be described in a following paper

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7,12 DMBA INDUCED RAT MAMMARY TUMOUR STUDIED FOR HORMONAL RESPONSIVENESS *IN VITRO*

2. *Organ Cultures*

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Aspegren, K. 7,12-DMBA induced rat mammary tumour studied for hormonal responsiveness *in vitro*. 2. Organ cultures. Acta path. microbiol. scand. Sect. A, 83: 37–50, 1975.

Specimens of DMBA-induced mammary tumour, DMBA-induced subcutaneous sarcomas, and virus induced sarcomas in the rat were incubated for 24–72 hours as organ cultures. The effect of various concentrations of testosterone, progesterone, and 17-β-oestradiol on cell survival and multiplication was measured as changes in incorporation of H^3-TdR into DNA. All three types of tumour reacted to the hormones. Statistical analysis of data demonstrated possible influence of factors other than hormone treatment. The specificity of hormone sensitivity assays *in vitro* is questioned.

Key words: Mammary tumours; rat; hormonal responsiveness; organ cultures.

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In vitro prediction of the hormonal responsiveness of an individual mammary carcinoma would be of great value in clinical practice. The DMBA-induced rat mammary tumour is hormone sensitive and has been much studied *in vivo* as a model tumour for man. A previous publication (2) studied the hormonal responsiveness *in vitro* of cell suspensions from this tumour and compared it with that of rat sarcomas induced chemically or with virus. Both classes of tumours showed the effects of added testosterone, progesterone, and oestradiol. As sarcomas *in vivo* are probably less influenced than mammary tumours by steroid hormones, the results could be a consequence of the rather artificial conditions of short term cell suspension cultures. Therefore, the work was repeated using organ tissue culture.

MATERIAL AND METHODS

Mammary tumours were induced in 50-day-old female outbred Sprague-Dawley rats (Anticimex, Stockholm) by intragastric instillation of 20 mg DMBA in oil suspension. Sarcomas were induced in similar rats by subcutaneous injection in the scapular region of 2 mg of DMBA in dimethyl sulphoxide. Virus sarcomas were the same as those used previously (2), i.e. one adeno-, one polyoma- and one Rous-virus induced tumour, each serially transplanted for at least 12 generations before use.

Only those sarcomas with growth confined to the back of the animal were used.

The animals were killed with ether. The tumours were cut out and immediately placed in culture medium at room temperature. They were then cleansed of areas of necrosis, if any, and cut into

small pieces with scissors. Six explants, approximately 1.5 mm square, were placed on a 9×6 mm strip of millipore filter (HAWG 047 0.45 μ, Millipore Inc., Bedford). The strips were laid on top of thin slices of cellulose acetate fabric (Spongostan® Ferrosan, Malmö). Two slices were placed in a D5 Carrel flask containing two ml of culture medium. The flasks were stoppered without further gassing and incubated at 37° C.

At preliminary experiments, Trowell's T8 culture medium (Bio-Cult, Glasgow) with 1 per cent human albumin added (KABI Stockholm) yielded better results than Parker 199 medium, and was therefore used throughout. The addition of 20 per cent human inactivated serum did not improve growth.

The following hormones (Sigma, St. Louis, Mo.) were used at the following final concentrations

Testosterone 0.5, 5 and 50 μg/ml
Progesterone 0.8, 8, and 80 μg/ml
17 β-oestradiol 0.02, 0.2 2, and 20 μg/ml

The hormones were dissolved in 96 per cent ethanol and added to the medium to give an ethanol concentration of 1 per cent. Control cultures were given ethanol only.

The medium contained 50 IU/ml of sodium benzyl penicilline and 50 μg/ml of streptomycin sulphate. The cultures were regularly checked for signs of infection. Throughout this work, infection was seen only twice. It was then randomly scattered among the flasks of an entire series and was plainly visible within 24 hours. These series were discarded.

Medium was changed every 24 hours. During the last four hours of culture the explants were transferred to conical centrifuge tubes with one millilitre of its own medium, and the culture was continued in submerged condition. To each tube was added 2 μCi tritiated thymidine (methyl-H^3-thymidine, spec. activity 1.9 Ci/mM, Schwartz/Mann Inc. Orangeburg). The cultures were terminated by washing once with cold phosphate buffer and were then treated with cold 5 per cent trichloro-acetic acid (TCA) for 30 minutes, washed once with TCA, and finally with absolute ethanol. The explants were stored at −20° C until further processed.

After thawing, the explants were dissolved in 1 ml of 1 N sodium hydroxide at 50° C for 3 hours. 100 μl of the solution was dissolved in 1 ml of Soluene 100 (Packard Ltd) and then mixed with 14 ml of scintillation fluid (300 mg dimethyl-POPOP and 5 g PPO in 1000 ml of toluene). Radioactivity of the samples was measured in a Packard TriCarb [illegible] spectrophotometer during 10 minutes counting. Efficiency was controlled by external standardization. The DNA content of the sample was estimated from two double determinations of 200 μl each using the previously described indole method (1).

Two methods of estimating H^3-TdR incorporation into DNA during the labelling period were compared. In one, radioactivity was directly expressed as $\log_e$ of the number of counts registered during ten minutes. In the other, the radioactivity was related to the DNA content of the culture with the expression

$$x = 100 \times \log_e \frac{\text{cpm} \times 10^4}{(\text{DNA}) \times \text{ext.std.}}$$

where (DNA) represents the "adjusted" DNA (1) expressed in arbitrary units and ext.std., the external standard count.

Pieces for histological sectioning were taken from each tumour when the cultures were set up. From the mammary tumours, randomly selected explants were also taken for each hormone concentration and each culture time. They were embedded in paraffin-wax, sectioned at 5 μ, and stained with H & E.

Statistical Methods

Hormone effects are evaluated as increases or decreases in thymidine incorporation in cultures with hormone added compared with parallely made cultures without hormones (controls). From a statistical point of view there are definite advantages in using data transformed to a logarithmic form, as the effects can then be described by differences instead of by ratios. In order to check whether logarithmically transformed data can be regarded as normally distributed, two experiments were performed where a tumour was cultured, without hormone addition, for 48 hours in 20 and 30 replicate cultures, resp. The distributions of the obtained variates as c.p.m. and as $\log_{10}$ c.p.m. were checked for normality by probit transformation: both were slightly skew and in opposite directions (coefficients of skewness for c.p.m. data was +0.60 and for log c.p.m. data was −0.58) but none deviated significantly from normality.

In order to evaluate the statistical significance of effects registered after hormone addition, the variance caused by hormone (= "between control and hormone-treated groups") was compared with the technical error variance between replicate cultures. Figs. 3–9 show the results graphically, where levels for $p = 0.05$, 0.01 and 0.001 are given. The error variance was estimated from a total of 415 double or triple replicate determinations. With the aid of Bartlett's test, these estimates were compared according to tumour group or hormone group. Replicate cultures that gave significantly deviating results were excluded from the calculation of the general error variance, this happened in 22 of the 415 determinations. When these were included in studies of hormone effects, correction was made



Fig 1 A Cross section of explant after 72 hours of culture. Millipore filter is at the bottom of the picture; the periphery of the explants is at the top. Central necrosis is plainly visible. Glandular arrangement of epithelial cells is partly preserved. Magnification × 140.

according to *Welch* (5). All were found non-significant.

Variance analysis was used in order to study the effect of hormones and also of other possible sources of variation: tumour (either individual tumour or kinds of tumours) and culture duration. The possible effect of these three sources of variation was studied as statistical interaction between them, both the primary interaction between them pairwise and the secondary interaction. Again, variances due to such interaction were evaluated against the general error variance.

RESULTS

Histological Examination

All tumours were either typical mammary carcinomas or sarcomas. The explants invariably showed signs of central necrosis after 24 hours. The extension of this necrosis did not increase with time, but signs of cell death were more pronounced at 72 hours than at 48 hours. The cells in the periphery of the explants always proliferated and there was no sign of cell death in this proliferative zone. Fig 1 shows typical examples of explants after 72 hours of culture.

Hormone treated cultures did not significantly differ in DNA content from corresponding control cultures, even at the highest hormonal concentrations and longest culture time. Thus the hormone addition probably did not reduce the number of surviving cells. No attempt was made to quantitate the histological findings.



Fig 1 B. Same as above at higher magnification (× 350). Field of view is limited to central area necrosis being seen at the bottom of the pl

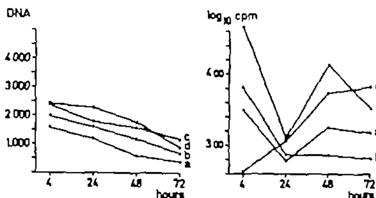


Fig 2 DNA values in arbitrary units (to the left) and H^3-TdR incorporation expressed as $\log_e$ cpm (to the right) of four mammary tumours (a-d) cultured with control medium for 4–72 hours. Each point represents the mean of three culture flasks.

Growth Characteristics

To study the behaviour of the culture system, four mammary tumours were cultured for 4 24 48, and 72 hours with triple flasks in each group Fig 2 gives the mean DNA and $\log_{10}$ cpm for these tumours. There is a steady decline in DNA content of the explants to about one third of the original value after 72 hours. This reasonably mirrors the above-described histological changes. The $\log_e$ cpm values decline in three tumours and rise in one tumour to scatter around 3 0 (1000 cpm) at 24 hours and then remain fairly steady (tumours a and b) or rise (tumour c and d) during the subsequent culture Apparently no correlation exists between total DNA of the explants and the incorporation of H^3 TdR into DNA.

Correction of Thymidine Uptake for DNA Content of Cultures

Our previous studies (1 2) using cell suspensions, evaluated thymidine incorporation as a so-called *a*-value i.e. c.p.m. per DNA in a log form (see above) DNA was used to estimate the number of cells in the test tube. As stated above in organ cultures as used in the present study only the margins of the explants proliferate and central necrosis develops. It was therefore necessary to investigate whether the correction for total DNA content of the cultures is necessary or even meaningful. It may increase the variability of the data if total DNA content is not correlated with the magnitude of the proliferating part of the explants. We therefore calculated the coefficient of variation (standard deviation in the distribution of replicate cultures as percentage of the mean) using data from the two large series previously described under the heading "Statistical methods" and using direct c p.m. values, $\log_e$ transformed c.p.m. values, and *a* values. The coefficient of variation was 5.3 per cent with $\log_{10}$ values, 20 6 per cent with *a* values, and 86 per cent with direct c.p.m. values. The log values thus gave the smallest variation and *a* values gave a clearly higher variation. There is thus no reason to try to correct for the DNA content of the explants. Throughout this investigation $\log_{10}$ c.p.m. values are therefore used.

Testosterone

Five different mammary tumours and six sarcomas (Figs. 3 and 4) were cultured at all three concentrations of hormone for 24–72 hours. Among the mammary tumours, four (Nos. 1–4) showed a similar dose-dependent response With 50 µg/ml, they were significantly inhibited at 24 hours. Increasing time showed a decrease in this response. At 72 hours, only one of the tumours was inhibited this effect may be random. The remaining tumour (No. 5) was markedly stimulated at 24 hours at all concentrations. At 48 hours, there was an inhibition at the highest concentration at 72 hours, both 5 and 50 µg/ml caused strong inhibition.

The control curves for the sarcomas differ more than those for the mammary tumours (Figs. 3 and 4) but all are of the same magni-

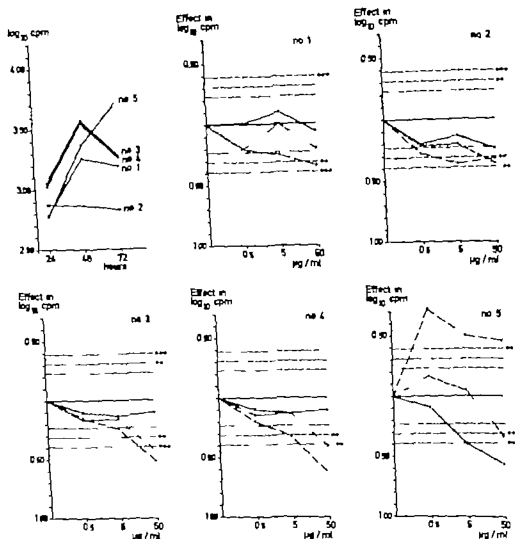


Fig 3. Results of culture of five mammary tumours with testosterone. Upper left insert gives mean log cpm of control flasks during 4–72 hours of culture for each tumour. Other inserts give dose-response curves for each culture time of individual tumours. Dashed/dotted lines (-·-·-) represent 24 hours of culture, dashed (- - -) 48 hours, and solid (——) 72 hours. Each figure gives significance limits of F-test between "hormone effect" and "general error". Crosses indicate points of measure that do not agree with the general error variance. None of these deviate significantly

* $0.05 > P > 0.01$ ** $0.01 > P > 0.001$ *** $P < 0.001$

tude. Three of the sarcomas (Nos. 6–8) were DMBA-induced primary sarcomas, whereas Nos. 9–11 were virus-induced and serially transplanted sarcomas. One virus sarcoma (No. 9) was not significantly affected by any concentration at any culture times. Another (No. 10) was stimulated by 5 µg/ml. The stimulation reached ** level at 72 hours, but was not significant at other times, although a trend towards stimulation with time was evident. At 50 µg/ml, there was an inhibition, although not significant, at 24 hours. The remaining sarcomas (Nos. 6–8 and 11) showed a rather uniform dose-response with increasing inhibition at increasing hormonal concentrations. There was no consistent variation of response with time. All four were significantly inhibited.

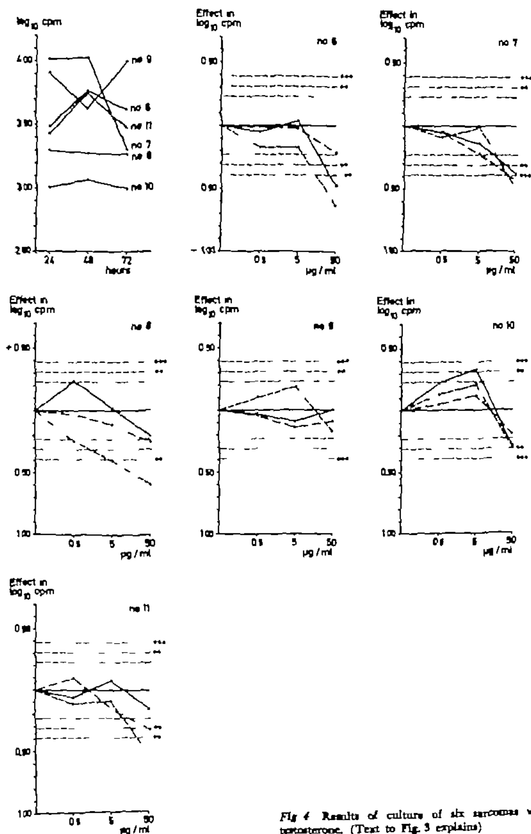


Fig 4 Results of culture of six sarcomas with testosterone. (Text to Fig. 3 explains)

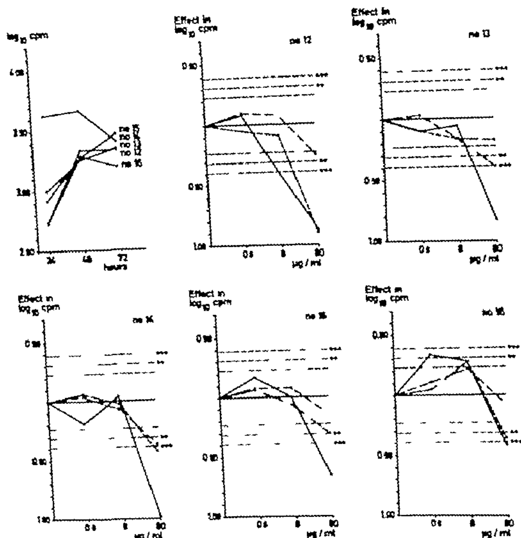


Fig. 5. Results of culture of five mammary tumours with progesterone. (Text to Fig. 3 explains)

To further analyse the responses, a variance analysis between the sources of variation "tumour" "hormone" and "culture time" was performed for the mammary and for the sarcoma group. Table 1 A and B give the result. In the case of the mammary tumours, there was significant secondary interaction between all three sources of variation. As regards the sarcomas, no such interaction was found. This probably means that the two groups behave differently to the treatment *in vitro*. Mammary tumours are affected in a more varied way than the sarcomas by testosterone.

Progesterone

Five mammary tumours were tested (Nos. 12–16). Fig. 5 gives the result. All tumours were inhibited by 80 μg/ml. The response increased with increasing time.

Tumour No. 16 differed from the previous four tumours, being significantly stimulated by 0.8 and 8 μg/ml at 72 hours and by 8 μg/ml at 48 hours.

Three DMBA induced primary sarcomas (Nos. 7, 8, and 17) and three virus-induced, serially transplanted sarcomas (Nos. 9, 10 and 18) were tested. Fig. 6 gives the result.

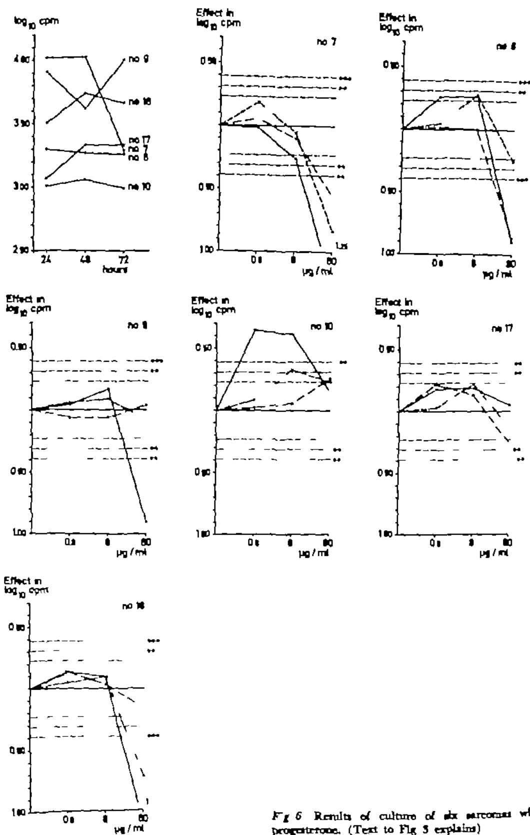


Fig 6 Results of culture of six sarcomas with progesterone. (Text to Fig 3 explains)

TABLE 1 *Secondary Interaction between Three Sources of Variation (Tumour : Hormone Treatment Culture Time) in Log CPM Values*

	Hormonal concentration	No. of tumours	df	Variance	F-value	P
A. Mammary tumours						
Testosterone	0.5	3	4	0.0962	3.70	***
	5	5	8	0.0400	2.37	*
	50	5	8	0.1206	7.14	***
Progesterone	0.8	5	8	0.0157	<1	NS
	8	4	6	0.0252	1.49	NS
	80	3	4	0.0135	<1	NS
Oestradiol	0.02	5	8	0.0247	1.46	NS
	0.2	5	8	0.0112	<1	NS
	2	4	6	0.0202	1.19	NS
	20	4	6	0.1049	6.21	***
B. Sarcomas						
Testosterone	0.5	6	10	0.0135	<1	NS
	5	5	8	0.0258	1.53	NS
	50	3	4	0.0037	<1	NS
Progesterone	0.8	5	8	0.0197	1.17	NS
	8	3	3	0.0000	<1	NS
	80	5	8	0.1479	8.76	***
Oestradiol	0.02	5	8	0.0587	3.48	***
	0.2	5	8	0.1010	5.98	***
	2	6	10	0.0821	4.86	***
	20	4	6	0.1217	7.21	***

F-values compare variance of secondary interaction with general technical error variance. General (technical) error variance = 0.0169 based on 471 df.

(Four of them were simultaneously cultured with testosterone see Fig 4)

One sarcoma (No. 17) was not significantly affected by the treatment except by 80 µg/ml at 24 hours this inhibition may be random. Three other sarcomas (Nos. 7 9 and 18) responded uniformly they were not affected by 0.8 and 8 µg/ml, but were strongly inhibited at 80 µg/ml and even more with increasing time. Sarcoma No. 8 was stimulated by 0.8 and 8 µg/ml at 72 hours and by 8 µg/ml at 48 hours, but inhibited by 80 µg/ml at all times. This sarcoma closely resembles mammary tumour No. 16 (Fig. 5) Sarcoma No. 10 showed a different reaction, as it was not inhibited but progressively stimulated with time. The largest stimulations were achieved by 0.8 and 8 µg/ml at 72 hours.

The analysis of interaction for the mammary group was not significant at any hormone concentration (Table 1 A) For the sarcomas, the two lower concentrations gave no interaction, but a highly significant interaction was found at the highest concentration. Thus there is no clear-cut difference in variability of response between the two different types of tumours.

17-β-Oestradiol

Five mammary tumours were cultured (see Fig 7) One of them, No 19 did not react with certainty Another No. 20 was inhibited by 20 µg/ml at 24 and 48 hours. Tumour No. 21 showed a tendency towards a biphasic reaction to the hormonal treatment, but this is not significant. No. 22 showed no reaction

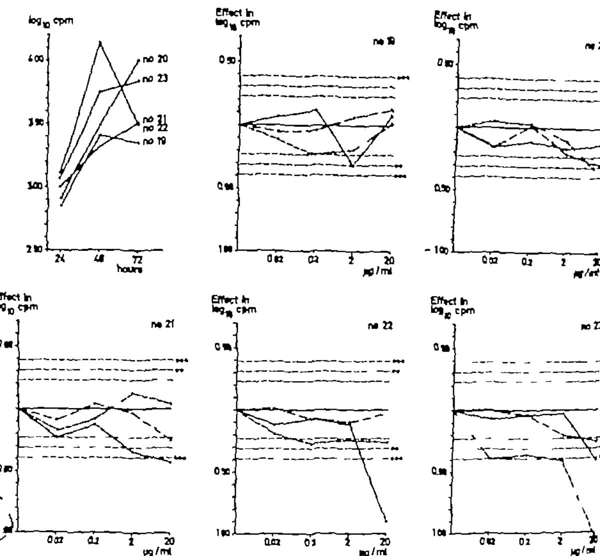


Fig 7 Results of culture of five mammary tumours with 17 β-oestradiol. (Text to Fig. 5 explains)

at 24 hours, a significant inhibition by all concentrations except the lowest at 48 hours, and again no reaction at 72 hours except by 20 μg/ml, which gave a strong inhibition. Tumour No. 23 showed a more clear-cut reaction in that 20 μg/ml gave inhibition at all culture times. Using the low concentrations, there was no response at 24 hours, inhibition at 48 and again no response at 72

Three DMBA induced sarcomas (Nos. 24–26) and three virus-induced and serially transplanted sarcomas (Nos. 27–29) were studied. Fig 8 gives the results. In the former group, one (No. 24) did not react except to one concentration at 48 hours. Sarcoma No. 25 was not affected at 24 hours, but was dose-response inhibited at 48. At 72 hours, it was affected in a biphasic manner the lowest concentration stimulated growth, the two middle concentrations gave no reaction, and the highest concentration again stimulated growth. Sarcoma No. 26 was inhibited at 24 hours, stimulated at 48, and did not react at 72. As the control curve shows, the difference of reaction between 24 and 72 hours cannot be explained by different level of control incorporation of these times. However at 48 hours, there is a decreased incorporation

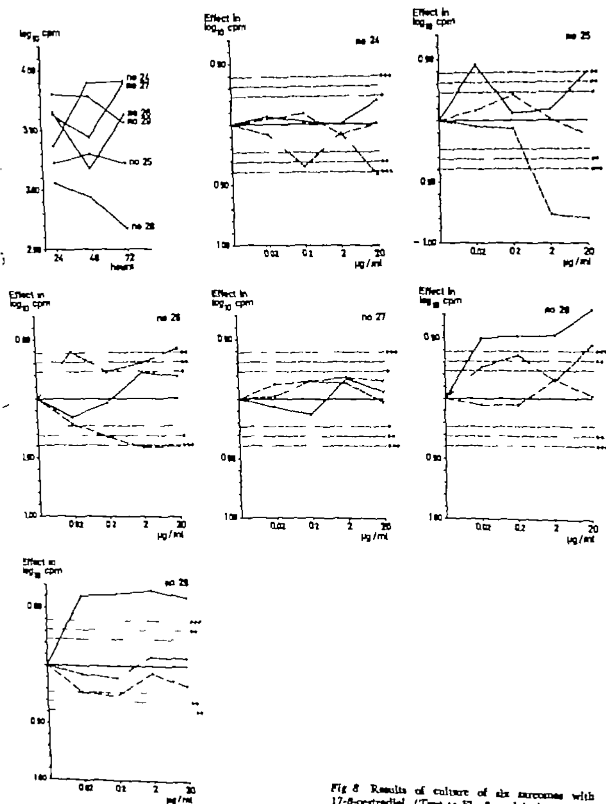


Fig 8 Results of culture of six sarcomas with 17-β-oestradiol. (Text to Fig. 5 explains)

of the controls this corresponds fairly well to the observed stimulation i.e. the effect of the hormone was to maintain the cultures at the 24-hour level.

Among the virus-induced sarcomas, No. 27 did not react. Sarcoma No. 28 did not react at 24 hours, was moderately stimulated at 48, and even more at 72. The results can be partly explained from the control curve which shows a declining incorporation of H^3 TdR with time, but the stimulation at 72 hours exceeds the corresponding decline of the control cultures. Sarcoma No. 29 was slightly inhibited by the two lower concentrations at 24 hours. At 48 hours, there was no significant response, but the curve shows a close resemblance to the 24 hour curve. At 72 hours, there was a marked stimulation at all concentrations of hormone. This is much above that to be explained by the decline in the control cultures and probably represents real stimulation.

The analysis of interaction was highly significant at all concentrations for the sarcoma group (Table 1 B). The mammary tumours showed no significant interaction if 0.02–2 μg/ml was used, but significant interaction at 20 μg/ml. Thus, sarcomas showed a more varied response than mammary tumours to oestradiol.

To summarize the results both types of tumours reacted to the hormone addition. Culture time and tumour individuality were important sources of variation. There was no consistent, "typical" difference in responses of mammary tumours and sarcomas (primary or serially transplanted).

As the effects seen in the two types of tumours were similar it was suspected that they could be caused by a technical error not included in the error between double or triple determinations, i.e. variation between experiments rather than within experiments. An effort was made to estimate this variation by the following experiments. One mammary tumour was set up as five simultaneous but separate experiments. Media, pipettes, glassware, and ethanol for the control medium were five different batches, but the tumour and the hormone solution (progesterone 80 μg/ml) were the same. Three control flasks and three flasks with hormone were set up in each of the five experiments and were cultured for 48 hours. The procedure was repeated using a virus-induced sarcoma.

The technical error variances of these experiments were tested for uniformity by Bartlett's test. As regards the mammary tumour it was not different from the technical error (general error) of the main material ($F = 1.01$ NS at 20 and 471 df) but in the case of the sarcoma it differed slightly ($F = 1.64$ $0.05 > P > 0.025$). Therefore, the technical errors of the experiments were used instead of general error for the F test comparing interaction and technical error (see below).

The five separate experiments performed with each tumour were tested for interaction between the possible sources of variation "experiments" and "hormone" and this was significant for both tumours ($F = 3.61$ for the mammary tumour and $F = 4.05$ for the sarcoma $0.025 > P > 0.01$). Thus there was obviously a source of interaction apart from the technical error which could be the cause of the observed variability in the hormonal effects.

The interaction variance between hormone effect and tumour (differences between experiments included) in the main material (mammary tumours and sarcomas) was 0.0943 i.e. larger than the interaction variance, 0.0763 found in the above-described experiments. Table 2 analyses this difference which is found to be statistically significant ($F = 8.0$ at 11 and 8 df $0.01 > P > 0.001$).

The contribution of the *different sources* of variation in these two tumours and the total material was estimated. As can be seen (Table 3 A, B and C) "hormone" dominates considerably "Technical error" and "experiment" are small, and "interaction between hormone and experiments" is negligible compared with "hormone".

From these considerations, it was judged that differences between different experiments and tumours may contribute to the observed effects, although this contribution

TABLE 2. *Analysis of Contribution to the Interaction between Hormone Effects and "Tumours"*

Source of variation	df	$\Sigma(X-\bar{X})^2$	Variance	F
Total	19	7.3161	–	
Between tumours	11	6.7059	0.6096	7.99**
Between "experiments" within tumours	8	0.6103	0.0763	

The latter source of variation is split into variation between "experiments" and true tumour variation.

TABLE 3. *Contribution of Different Sources of Variation to the Total Recorded Variation*

Source of variation	df	Estimate of σ^2	Percentage of total estimated σ^2
A.			
Total	29	0.7862	–
Experiment	4	0.0055	0.7
Hormone	1	0.7191	91.5
Interaction between hormone and experiment	4	0.0446	5.7
Technical error	20	0.0171	2.2
B.			
Total	29	3.4971	–
Experiment	4	0.0025	0.1
Hormone	1	3.4388	98.3
Interaction between hormone and experiment	4	0.0281	0.8
Technical error	20	0.0277	0.8
C.			
Total	39	0.2975	–
Experiment & tumour	9	0.0063	2.2
Hormone	1	0.2356	79
Interaction between hormone and experiment	9	0.0387	13.0
Technical error	20	0.0170	5.7

A Simultaneously performed "experiments" from one mammary tumour 48 hours culture time, progesterone 80 μg/ml.
B Simultaneously performed "experiments" from a polyoma virus induced sarcoma 48 hours culture time, progesterone 80 μg/ml.
C Total tumour material 48 hours culture time progesterone 80 μg/ml.

must be small if the hormonal effects are large.

DISCUSSION

An earlier paper (2) used short term incubations of cell suspensions to study the responsiveness of rat mammary tumours and sarcomas to steroid hormones. Effects were found in both types of tumours, measured as incorporation of H^3 TdR into DNA. Because the short incubation time used may not permit the estimate of "specific" hormone effects via a receptor mechanism, the present investigation was performed on the same type of tumours using organ tissue cultures for up to three days. A similar study was made by *Welsch & Rivera* (6) who cultured explants

of DMBA rat mammary tumour for five days and studied the influence of prolactin and oestradiol. The present investigation confirmed their results obtained by oestradiol. But when rat sarcomas, both primary and serially transplanted, were used as controls in the present investigation, they also reacted to the hormones, and this 'reaction' was probably not caused by technical artefacts.

Murakami et al. (4) investigated the effect of castration, stilboestrol treatment, and castration + stilboestrol on DMBA induced subcutaneous sarcomas in male rats. They found the incidence of the tumours unaffected, which is in contrast to the DMBA induced mammary tumour (3). However the growth of the tumours was affected: the hormonal treatment increased their size.

Unfortunately their report does not give enough data from the experiments to permit statistical evaluation. It was also performed on non-hypophysectomized animals: thus an influence of pituitary hormones on the sarcomas cannot be excluded.

As the incidence of sarcomas induced by DMBA is unaffected, this tumour should at least be less sensitive to steroids than the mammary tumour. Despite this, both the previous (2) and the present study demonstrate *in vitro* reactions to added hormones of the tors (6) and the effects cannot be explained as merely unspecifically toxic effects because different hormones have different effects on the same tumour (2) and stimulations can be seen at the highest concentrations used. Thus the results of studies of this type must be evaluated with great caution as regards specific effects of steroid hormones.

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EXPERIMENTAL MURINE LEPROSY

3 *Early Local Reaction to* Mycobacterium lepraemurium *in C3H and C57/BL Mice*

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Closs, O & Haugen, O. A. Experimental murine leprosy 3 Early local reaction to *Mycobacterium lepraemurium* in C3H and C57/BL mice. Acta path. microbiol. scand. Sect. A, 83 51–58, 1975

M lepraemurium was injected subcutaneously into two inbred strains of mice, C3H and C57/BL, in order to study the local reaction at various time intervals. Within 6 hours an acute inflammatory reaction developed at the site of injection. In the course of the following days it was replaced by a mononuclear infiltrate. The influx of mononuclear cells appeared to be somewhat greater in C57/BL than in C3H mice. Apart from this, little difference was observed between the two strains until at 4 weeks when a vigorous granulomatous reaction developed in the C57/BL strain. This reaction apparently arrested further local spread of the infection. The histological appearance of the infiltrate indicated that a delayed hypersensitivity reaction was taking place. No signs of such reaction was observed in the C3H strain.

Key words *Mycobacterium lepraemurium* mycobacterial infection inflammation hypersensitivity delayed.

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Resistance to mycobacterial infection depends on the capability of the host to mount a cell mediated immune (CMI) reaction (*Mackaness* 1971) We have previously demonstrated that outbred mice vary in their host response against *Mycobacterium lepraemurium* (MLM) (*Closs & Haugen* 1973 1974a) Mice of an inbred strain all respond in a rather similar manner to MLM-infection, but significant differences may be observed between various inbred strains (*Closs & Haugen* 1974 *Kawaguchi* 1959) Our previous work was based mainly on the study of lepromas developing in the liver after intravenous (iv) or intraperitoneal (ip) inoculation. The inbred strains C3H and B57/BL seem to form a useful model for the study of host resistance in murine leprosy in C3H mice there is no efficient host reaction against the bacilli which show unlimited growth, while in C57/BL the macrophages that contain bacilli are surrounded by a lymphocytic reaction, and unless a too large inoculum is given these animals may be capable of controlling the infection.

Local accumulation of lymphocytes is probably required for macrophage activation (*Dannenberg* 1968, *Mackaness* 1968) as an expression of cell mediated immunity Since iv or ip inoculation gives rise to a large number of infectious foci the number of specifi-

4

cally activated T-cells available per lesion may become too small to obtain maximum effect of the CMI response. High levels of resistance may therefore be more easily established in a model based on local infection.

Kawaguchi (1959) classified murine leprosy into a benign and a malignant form during a study of subcutaneous lepromas on the thorax. We wanted to investigate this work further by studying MLM infection in the two mouse strains C3H and C57/BL. This paper describes the development of the local reaction in the first 4 weeks after inoculation, a second paper (*Closs & Haugen* 1975) describes the subsequent development of the lepromas.

MATERIAL AND METHODS

Experimental animals. Specific pathogen free (spf) female mice of the inbred strains C3H/A and C57/BL/6J were obtained from Gl. Bomholtgård Ltd. Denmark. The animals weighed 16–18 grams at the beginning of the experiment. They were kept in cages, 6–8 mice in each, fed on pellets (Norwegian Standard Stock No. 1 Mice and Rats) and allowed tap water *ad libitum*.

Propagation of bacilli. M. lepraemurium (MLM) Douglas strain were propagated in the outbred strain NMRI and harvested as previously described (*Closs & Haugen* 1974)

Counting of bacilli. Counting of bacilli (*Hart & Rees* 1960) was performed as previously described (*Closs & Haugen* 1974)

Inoculation procedures. Before inoculation all hair covering small area on the right side of the thorax was removed using Surgex® Hair Remover Cream (Tilder-Cates Laboratories Inc., Wayne, N.J., U.S.A.) Using a 1 ml disposable syringe fitted with a 27 gauge needle between 20 and 30 microliters of a bacillary suspension containing 5×10^{9} bacilli per ml were deposited just beneath the epidermis in such a way as to produce a visible, localised swelling

Histological examination. At certain time intervals 2 animals of each strain were killed and tissues removed for histological examination. Tissue specimens were fixed for approximately 12 hours in formol alcohol containing 5 per cent glacial acetic acid, and then transferred to 80 per cent ethanol. Further processing and embedding in paraffin was performed according to standard procedures. Sections were stained with haematoxylin and eosin (HE) and, for the demonstration of acid fast bacilli, by the Ziehl-Neelsen (ZN) technique.



Fig. 1. Small abscess containing polymorphonuclear leucocytes and mononuclear cells 48 hours after subcutaneous injection of 1.5×10^{8} *M. lepraemurium*. C57/BL strain. HE × 125.

RESULTS

Local reaction 6 hours after inoculation. Within 6 hours of the subcutaneous inoculation of C3H and C57/BL mice an acute inflammatory reaction developed at the injection site. The histological findings were essentially the same in both strains an infiltrate consisting mainly of polymorphonuclear leucocytes (PML) was located in the subcutaneous fatty tissue and in addition, a number of mononuclear cells with various amounts of cytoplasm and a kidney-shaped nucleus were observed. The latter cells had the appearance of macrophages or blood borne monocytes. Small lymphocytes or plasma cells were not present. Acid fast bacilli were seen both intra and extracellularly the majority of phagocytized bacilli appearing within PML.

Local reaction 24 hours after inoculation. The infiltrate had increased in size and become more distinct showing the histological features of a small abscess situated subcutaneously at the surface of the underlying muscle (Fig. 1) In C57/BL mice the infiltrate still contained many PML, most of which were intact but now the predominant cell type was the mononuclear phagocyte. At this stage most of the bacilli seemed to be confined to the latter cell type It was also noted that mononuclear cells were more numerous at the periphery of the infiltrate

indicating that an influx of such cells was taking place. In C3H mice the infiltrate contained much more cellular debris, probably representing dead and fragmented PML. Intact cells of this type were rarely encountered (Fig. 2) and bacilli were mostly found extracellularly.

Local reaction 48 hours after inoculation. The cellular infiltrate contained the same cell types as after 24 hours, but now there was a greater predominance of actively phagocytizing mononuclear cells. The infiltrate seemed to spread as a thin band of phagocytes in the subcutaneous tissue spaces and between the connective tissue septa of the underlying muscle. Although some intact PML containing acid fast material were still found, nearly all intracellular bacilli were now within macrophages. In C3H mice, the influx of macrophages appeared to be less than in C57/BL, and in some areas groups of macrophages were seen apparently filled to the limit of their capacity since an abundance of free bacilli were present in the vicinity (Fig. 3A). In the C57/BL mice free bacilli were rarely encountered. Although single macrophages completely filled with bacilli were frequently encountered they were usually surrounded by others containing only a few bacilli (Fig. 3B). It therefore appears that MLM is more effectively taken up by the reticuloendothelial system in C57/BL than in C3H mice.

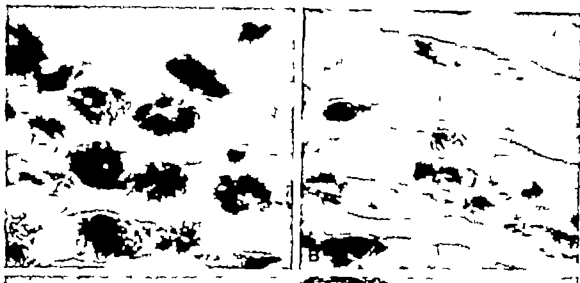
Local reaction 1 and 2 weeks after inoculation. In both strains the infiltrate now appeared as a stretched-out band consisting purely of macrophages and situated deeply in the subcutaneous fatty tissue. The macrophages observed in C57/BL were rounded or elongated, mainly with basophilic cytoplasm, and contained moderate to large amounts of bacilli (Fig. 4A). In the infiltrate of C3H mice many of the macrophages were enlarged, apparently distended by the great amount of acid fast material they contained (Fig. 4B). In the HE-stained preparation the cytoplasm of these cells appeared pale. Extracellular bacilli were rare at this stage. Notably up to this point 2 weeks after inoculation, no signs of lymphocytic infiltration were noticed in either of the strains.



Fig. 2. Abscess in the C3H strain formed 48 hours after subcutaneous injection of 1.5 × 10⁸ *M. lepraemurium*. Note the amount of cellular debris and the relative paucity of mononuclear cells. HE × 325.

Local reaction 4 weeks after inoculation. About 4 weeks after inoculation the lesions of the C57/BL animals were dramatically altered: the infiltrate became much larger and showed striking oedema (Fig. 3C). Areas of necrosis were found in the central part of the lesion, containing disintegrating macrophages, cellular debris and PML. In this area there was also pronounced necrosis of collagen fibres. In the outer zone the infiltrate was rich in mononuclear cells, having both monocytes and small lymphocytes present. The latter cell type often occurred in small clusters, sometimes in close relation to blood vessels. Occasionally plasma cells were also found. The infiltrate extended into the epidermis and was seen to surround skin appendages (Fig. 5). The cellular reaction also spread to the underlying muscle, forming a pleomorphic infiltrate between the muscle bundles.

The central area of the lesion contained numerous scattered bacilli, nearly all extracellular and often appearing only faintly stained (Fig. 3D). At the periphery of this central necrotic area there was a circular zone consisting of macrophages which had ingested considerable amounts of acid fast bacilli, leaving very few in the extracellular space (Fig. 3E). In the most peripheral parts


B




D
E
F
G

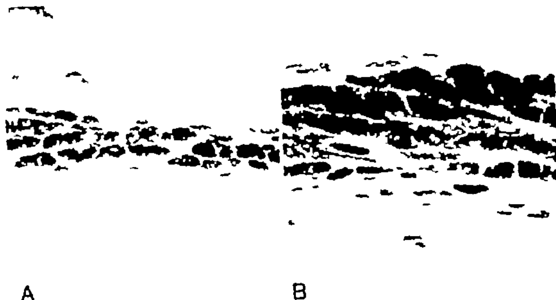


Fig. 4 Subcutaneous band of cells containing acid fast bacilli 2 weeks after inoculation of mice with *M. lepraemurium*. A. C57/BL strain. Macrophages of normal size containing moderate to large amounts of bacilli. ZN × 300. B. C3H strain. Greatly distended macrophages containing very large amounts of bacilli. ZN × 300.

Fig. 3 A and B. Cells containing acid fast bacilli 48 hours after subcutaneous injection of *M. lepraemurium*. A. C3H strain. Note the presence of extracellular bacilli and the lack of empty macrophages. B. C57/BL strain. Note the presence of many macrophages containing few or no bacilli. No extracellular bacilli are seen. ZN × 1000.

C. Granuloma forming in the C57/BL strain, 4 weeks after subcutaneous injection of 1.5×10^{8} *M. lepraemurium*, showing central necrosis and presence of several zones in the surrounding infiltrate. Note oedema in the adjacent subcutaneous tissue. HE × 25.

D, E and F. C57/BL strain 4 weeks after inoculation. Amount of acidfast bacilli in the granuloma at 3 different levels. D. Central necrotic area. No intact cells are seen and all bacilli are extracellular. E. Macrophage zone at the periphery of the necrotic area showing large amounts of intracellular bacilli. F. Peripheral zone. Bacilli are scarce. ZN × 1000.

G. The lesion of C3H mice 4 weeks after subcutaneous injection of 1.5×10^{9} *M. lepraemurium* consisting of a uniform population of large macrophages densely filled with bacilli. ZN × 1000



Fig. 5 Cellular reaction involving the subepidermal tissue. C57/BL strain 4 weeks after subcutaneous injection of 1.5×10^{9} *M. lepraemurium*. HE × 210.

of the infiltrate only a few scattered bacilli were seen, and the majority of the macrophages did not contain any acid fast material (Fig 3F) The vigorous reaction occurring in C57/BL mice may inhibit further spread of the infection in this strain.

In contrast to this, the lesions of C3H mice remained unchanged except for an increase in size. The infiltrate consisted of rounded or polygonal, stretched out macrophages forming a slender subcutaneous infiltrate. All these cells were packed with bacilli so that there was no evidence of an invasion of excess mononuclear cells (Fig 3G) Polymorphonuclear leucocytes were seen in a localized area of one infiltrate.

DISCUSSION

The present investigation has shown that MLM injected subcutaneously into mice will give rise to an acute inflammatory reaction with local oedema and accumulation of *many polymorphonuclear leucocytes* (PML) and monocytes. The reaction, which is similar to the early inflammatory reaction to *M tuberculosis* (*Forwald* 1932 *Miles* 1955) other bacteria (*Burke & Miles* 1958) and non-living substances (*Paz & Spector* 1962) demonstrates that MLM acts as an irritant, and indicates that it is chemotactic for blood leucocytes. Regarding these early manifestations of the infection virtually no difference was observed between the two mouse strains C3H and C57/BL.

Although MLM initially is taken up by the PML, within 24 hours most of the bacilli have been transferred to mononuclear phagocytes were they remain The outcome of the infection will therefore depend on the fate of MLM within these cells.

The influx of mononuclear cells into the area of inflammation apparently occurred at a higher rate in C57/BL than in C3H mice. The relatively few mononuclear phagocytes that were observed in the lesions of C3H mice during the first 48 hours were not able to phagocytose all the bacilli present. As a result, MLM remained extracellular for longer in this strain in comparison to the C57/BL mice. Since MLM is an obligate intracellular parasite it is not likely that this delay in phagocytosis contributes to the more severe course of MLM infection in the C3H strain.

In the course of the first 48 hours the macrophages in the lesions of C3H mice all became rather heavily loaded with MLM compared to the situation in C57/BL mice where the macrophages contained various amounts of acid fast bacilli. The lag in phagocytosis observed in the C3H strain can, therefore, be attributed to an insufficient influx of mononuclear cells and not to inability of the individual phagocyte to ingest the MLM.

About 4 weeks after inoculation the appearance of the local lesion changes abruptly *in C57/BL mice. There is a large influx of* cells forming a typical dermal granuloma at the surface of the underlying muscle. The predominance of mononuclear cells in the infiltrate both macrophages and lymphocytes, indicates that a delayed hypersensitivity reaction is taking place (*Vasalli & McCluskey* 1971) Similar changes were not observed in the C3H mice. Except for a moderate increase in size the lesions in these animals remained unchanged, not exhibiting any signs of an altered host reaction.

Since every macrophage in the C3H lesions seemed to be quite full of bacilli and the influx of new phagocytes apparently was very little we considered the possibility that these lesions might grow by local division of bacilli laden cells. However in spite of extensive examination of a number of sections, including sections from colchicine treated animals, mitoses in macrophages were never observed (*Closs & Haugen* unpublished observations) Our failure to demonstrate mitoses does not exclude the possibility that these cells divide. Several workers have reported that division of macrophages occurs in the local lesions of BCG infected animals (*North* 1969 *Ando et al.* 1972) but found that macrophage proliferation seems to coincide with activation of the macrophage. These observations may

therefore have no direct bearing on the situation in our C3H mice where activation of the macrophages does not seem to take place.

In C57/BL mice it takes about 4 weeks of MLM infection to develop a delayed hypersensitivity reaction. This agrees with the findings of *Kubica et al.* (1973) who found that in CD-1 mice infected with various species of mycobacteria a delayed hypersensitivity reaction could be evoked in the footpad 3-4 weeks after inoculation. A state of delayed hypersensitivity has been found to develop early during an infection with *Listeria monocytogenes* but later during a BCG infection, indicating that the development of these components of the host response is determined by the metabolic characteristics of the parasite (*North* 1969). On the other hand, *Gray* (1960) investigating natural resistance to tuberculosis amongst inbred mice, concluded that differences in the susceptibility of these mice to the infection were due to variations in the speed at which they acquired immunity. It thus appears that the time needed to develop cell mediated immunity to a certain infection is dependent on properties of the parasite as well as on factors of the host.

In C57/BL mice the number of bacilli per macrophage, although different in separate areas of the lesion, falls sharply after 4 weeks indicating that rephagocytosis of the organisms is taking place. This may be due to spontaneous rupture of the macrophages caused by bacterial multiplication. Such extracellular spread of antigen could be the necessary stimulus for a cell mediated immune response. On the other hand, destruction of bacilli-containing macrophages may be the result of an immune reaction caused by the local accumulation of lymphocyte specifically sensitized to MLM antigens. Thus, the lag period from inoculation to the onset of the local granulomatous reaction in C57/BL mice may either reflect the time it takes to develop systemic cell mediated immunity to MLM or the time needed for the infection to progress to a point where it produces the appropriate stimulus for a CMI reaction. The present study does not permit any definite conclusions regarding this problem.

Based on the present observations the local reaction occurring after subcutaneous inoculation of murine leprosy bacilli may be divided into three stages: 1) Acute inflammation, 2) compatible phase, and 3) granuloma formation. All three stages may be observed in C57/BL mice, while a granulomatous reaction does not seem to develop in C3H mice. In addition, there appears to be an insufficient accumulation of macrophages in the early lesion of C3H mice.

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EXPERIMENTAL MURINE LEPROSY

4. *The Gross Appearance and Microscopic Features of the Local Infiltrate After Subcutaneous Inoculation of C3H and C57/BL Mice with* Mycobacterium lepraemurium

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Closs, O. & Haugen, O. A. Experimental murine leprosy. 4. The gross appearance and microscopic features of the local infiltrate after subcutaneous inoculation of C3H and C57/BL mice with *Mycobacterium lepraemurium*. Acta path. microbiol. scand. Sect. A. 83: 59–68, 1975.

Mice of the inbred strains C57/BL and C3H were inoculated subcutaneously on the thorax with *M. lepraemurium*. In C57/BL mice a firm, raised, sharply defined nodular infiltrate developed 4 weeks afterwards, while in the C3H strain the infection produced a soft, flattened infiltrate with ill-defined margins, which did not become palpable until 10 weeks after inoculation. A limited spread of the infection occurred early in both strains, but apparently multiplication of the microorganisms was very restricted in C57/BL mice; progressive, disseminated growth of the bacilli was observed in the C3H strain only. In C57/BL mice the granulomatous reaction, developing 4 weeks after inoculation and leading to abscess formation, ulceration and scar formation, apparently inhibited both local multiplication and further spread of the bacilli. In C3H mice no host reaction was detected and the bacilli appeared to grow unrestrictedly. In some C57/BL animals, decrease in host resistance occurred during the infection, causing reactivation of the local lesion and an apparently rapid proliferation of bacilli. Observations regarding the lesions in superinfected animals indicated that a systemic immune reaction develops in the C57/BL strain about 4 weeks after inoculation, whereas this does not occur in the C3H strain.

Key words: Mycobacterium lepraemurium; mycobacterial infection; inflammation; hypersensitivity, delayed.

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In a previous paper (*Closs & Haugen* 1975) we have described the early reaction to *Mycobacterium lepraemurium* (MLM) after subcutaneous injection in the two inbred mouse strains C3H and C57/BL. The present study is a continuation of that investigation. It includes a study of the macroscopic features of the lepromas that develop in the two strains after primary infection and superinfection, and also, histological examination of the lesions at various time intervals later than 4 weeks after inoculation.

MATERIAL AND METHODS

Experimental animals. Specific pathogen free (spf) female mice of the inbred strains C3H/A and C57/BL/6J were obtained from Gl. Bomholtgård Ltd., Denmark. The animals weighed 16–18 grams at the beginning of the experiment. They were

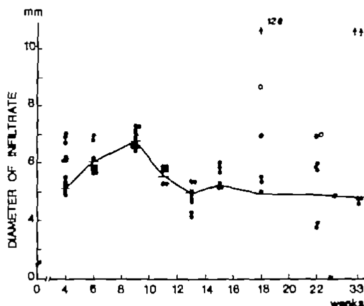


Fig. 1. The size of the local infiltrate after subcutaneous inoculation of C57/BL mice with 1.5 × 10 *M. lepraemurium*. A line has been drawn between the arithmetric mean of each group. Open circles represent lesions in which the reaction has become reactivated. These values were excluded before the line was drawn.

kept in cages, 6–8 mice in each, fed on pellets (Norwegian Standard Stock No. 1 Mice and Rats) and allowed tap water *ad libitum*.

Propagation of bacilli. M. lepraemurium (MLM) Douglas strain, were propagated in the outbred strain NMRI and harvested as previously described (*Closs & Haugen* 1974)

Counting of bacilli. Counting of bacilli (*Hart & Rees* 1960) was performed as previously described (*Closs & Haugen* 1974)

Inoculatio procedures. Inoculation of bacilli followed the description given in the preceding paper (*Closs & Haugen* 1975) 30 microlitres of bacillary suspension being injected into each animal.

Histological methods. Tissue specimens were fixed for approximately 12 hours in formol alcohol containing 5 per cent glacial acetic acid, and then transferred to 80 per cent ethanol. Further processing and embedding was performed according to standard procedures. Sections were stained with haematoxylin and eosin (HE) and for the demonstration of acid-fast bacilli, by a modified Ziehl-Neelsen (ZN) technique (*Putt* 1951)

Inspection of the ject on st and measurement of lepromas. The site of injection was inspected at weekly intervals and any palpable tumour or visible ulceration recorded. The diameter of the palpable infiltrates was measured using an engineer's micrometer which allowed readings in 1/10 millimetres. The micrometer was held in such way that the scal could not be read until after each measurement was finished. Since the lesions in C3H mice were diffused soft and not well defined, they were more difficult to measure accurately than the nodular infiltrates arising in the C57/BL strain. Because of the different shape of the infiltrates the measurements in the two strains are not directly comparable.

Statistical methods. Wilcoxon signed rank test (*Snedecor & Cochran* 1967) was used to test differences between groups of measurements. Values of p below 0.05 were regarded as statistically significant.

RESULTS

Macroscopic development of lepromas. No changes were observed at the site of injection in either of the two strains until about 4 weeks after inoculation with 1.5 × 10[8] MLM. At that time a sharply demarcated, raised, firm nodule became palpable in the C57/BL strain. During the following weeks the diameter of the tumour varied as shown in Fig. 1. After 6–8 weeks hair was lost from the skin covering the lesions and after the 10th week of infection most of the lesions ulcerated, followed by the discharge of thick, yellowish pus. The ulcerated lesions later became flattened and acquired a scar like appearance. After about 15 weeks the lesions were of two different types the stable lesions and those in which the infection had become reactivated making them increase in size. When such reactivation occurred, satellite lesions having a softer consistency than the

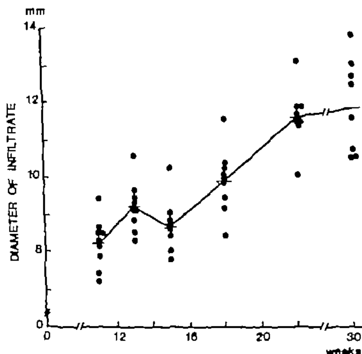


Fig. 2 The size of the local infiltrate after subcutaneous inoculation of C3H mice with 1.5 × 10⁸ *M. lepraemurium*. A line has been drawn between the arithmetric mean of each group.

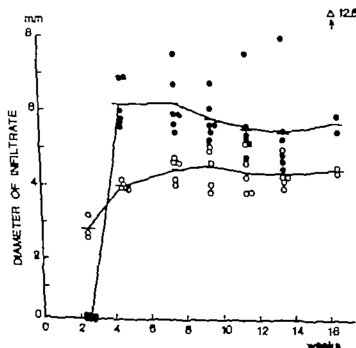


Fig. 3 The size of infiltrates after subcutaneous inoculation of 2 groups of C57/BL mice with 1.2 × 10⁸ *M. lepraemurium* (●) primary infection, (○) superinfection. The lines have been drawn between the arithmetic mean of each group. One value (Δ) was excluded before the lines were drawn. After 14 weeks 3 animals of each group were killed for histological examination.

primary lesions, could develop quickly on the thorax or in the axilla.

I C3H mice there was no palpable infiltrate at the injection site until about 12 weeks after inoculation when a smooth, flat tened tumour with ill-defined margin developed, increasing slowly but steadily in size until termination of the experiments at 30 weeks (Fig. 2). The small peak occurring at 13 weeks in Fig. 2 is probably an artefact

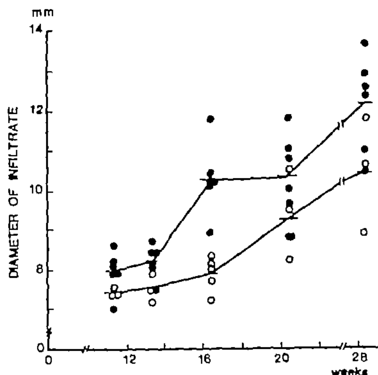


Fig 4 Size of infiltrates after subcutaneous inoculation of 2 groups of C3H mice with 1.2 × 10⁸ *M lepraemurium* (●) primary infection, (○) superinfection. The lines have been drawn between the arithmetic mean of each group.

due to difficulties in defining the edge of the lesion at this stage. Ulceration was not observed and loss of hair was moderate and occurred late.

Development of the local lesion following superinfection. An equal dose of bacilli (1.2 × 10⁸) was injected subcutaneously into normal animals (primary infection) and into animals inoculated on the other side of the thorax 1½ weeks previously (superinfection). In the superinfected animals of the C57/BL strain, the lesions developed simultaneously on both sides of the thorax, while there was as yet no palpable infiltrate at the site of inoculation in the control group.

In C57/BL mice the superinfected animals developed a palpable infiltrate after 2½ weeks (Fig 3). During the whole period of observation the lesions produced by superinfection remained smaller than those observed in animals with primary infection. The lesions arising from superinfection usually did not ulcerate, but in the skin covering the infiltrate most of the hairs disappeared.

No measurable infiltrates were present in C3H mice until almost 12 weeks after inoculation (Fig 4). Although the infiltrates tended to be smaller after superinfection than after primary infection this difference was not statistically significant except at one point, i.e. after 16½ weeks. The edge of the infiltrate was difficult to define in this strain and the diversion in the upper curve occurring at 16½ weeks probably is due to an error of measurement.

Local reaction 6 and 10 weeks after inoculation. In C57/BL mice the infiltrate appeared definitely larger than after 4 weeks (cf Fig 1). Microscopic examination revealed that extensive central necrosis was still present, and in some areas the cellular debris was infiltrated by polymorphonuclear leucocytes (PML). Oedema was less marked than before. The major part of the infiltrate consisted of mononuclear cells, both lymphocytes and macrophages. Most of the lymphocytes were seen diffusely infiltrating the outer part of the lesion, while some formed small aggregates, often in close relation to blood vessels. In the outer parts of the infiltrate cells

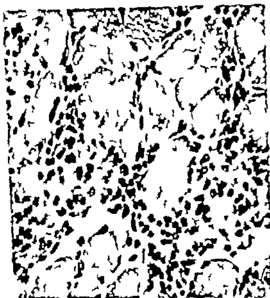


Fig 5 Clusters of epithelioid cells among fibres of striated muscle. C57/BL mouse 10 weeks after subcutaneous inoculation with 1.5×10^6 *M lepraemurium* HE × 360.

also occurred which closely resembled epithelioid cells. After 10 weeks the lesions showed a striking increase in such cells and, together with fibroblasts, they dominated the outer zone of the lesion (Fig 5) It appeared as if the central parts of the infiltrate, which contained most of the acid fast material (Fig 6) gradually became shielded off from the outer part of the granuloma by layers of collagen and fibrous tissue. At 10 weeks a relatively sharp demarcation line had formed between the macrophages of the inner zone and the reactive cells surrounding them. In the outer zone bacilli were scarce and were always found intracellularly Whereas bacilli in the central part of the lesion appeared as solid staining rods, bacilli in the outer zone showed many elongated forms which were often faintly and irregularly stained. In C3H mice the lesions showed no signs of oedema. Compared with the situation 4 weeks after infection (*Closs & Haugen* 1975) the macrophageband in the subcutis had increased in thickness. The infiltrate was composed of a remarkably uniform population of cells heavily loaded with MLM and the influx of "virgin phagocytes" appeared to be minimal. Lymphocytes were rarely found. Occasional groups of PML and plasma cells were observed between the macrophages.

Observations on the late development of the infiltrate From 10 to 15 weeks after inoculation abscess formation progressed in the C57/BL strain and in some of the lesions definite signs of ulceration of the dermis were found microscopically The granulation tissue now appeared more fibrous and contained many fibroblasts. Lymphocytes were still numerous and, occasionally multinucleated giant cells were found (Fig 7)

After 15 weeks the infection was more varied in C57/BL mice regarding the development of the local lesion. In most of the animals in which ulceration had occurred the lesion contained less acid-fast material and apparently the infection was effectively controlled. The lesions found in animals with reactivated infection consisted of large homogeneous aggregates of macrophages con-



Fig 6 Subcutaneous granuloma in a C57/BL mouse 6 weeks after inoculation with 1.5×10^6 *M lepraemurium*. Three zones are shown (n) necrotic area in the centre, (m) annular zone of macrophages containing much acid fast (dark) material, and (p) peripheral part, containing reactive cells and little acid-fast material. Note the relatively sharp demarcation between the zones (m) and (p) Modified Ziehl-Neelsen, × 25

taining many bacilli (Fig 8) Lymphocytes and other reactive cells were scarce and thus the lesions were more like the typical lesions found in C3H mice.

As the infection progressed, the lepromas expanded slowly but steadily in C3H mice until towards the end of the observation period they occupied the whole dermis and also infiltrated the underlying muscle. Lymphocytes were scarce, and there was no zone of reaction around the cells containing bacilli. Clusters of plasma cells were frequent (Fig 9) Definite signs of ulceration were not found, but the epidermis showed atrophy and atrophic changes were also present in the skin appendages, thus accounting for the observed loss of hair

Spread of the infection. In both strains bacilli could be demonstrated in the regional lymph nodes 48 hours after inoculation using auramin rhodamin stained preparations (unpublished observation) Definite signs of macrophage accumulation were not evident until 4 weeks later when small eosinophilic nodules were seen in the peripheral area of

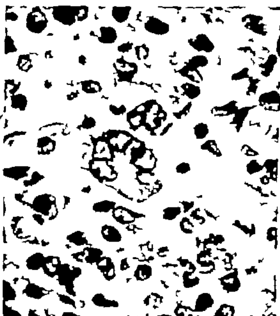


Fig 7 Multinucleated giant cell in a granuloma from a C57/BL mouse 15 weeks after subcutaneous inoculation with 1.5×10^{8} M. lepraemurium. HE $\times$ 920.



Fig 8 Satellite lesion involving subdermal muscle, developing after reactivation of *M lepraemurium* —infection in a C57/BL mouse 22 weeks after inoculation. The dark areas correspond to macrophages containing large amounts of acid-fast material. Note the lack of reaction around these cells. Modified Ziehl-Neelsen staining, $\times$ 230.

TABLE 1 *The Spread of Infection to Various Organs 30 to 33 Weeks After Subcutaneous Inoculation of Mice with* M. lepraemurium

Organ	Mouse strain			
	C57/BL		C3H	
	G	*B*	*G*	*B*
Liver	7/7	5/7	9/9	8/9
Spleen	3/7	3/7	8/9	9/9
Thymus	6/7	6/7	3/9	3/9
Lungs	1/6	0/6	4/7	4/7
Heart	0/7	0/7	1/9	1/9
Skeletal muscle	0/7	0/7	0/9	0/9
Kidneys	0/7	0/7	0/8	0/8
Adrenal glands	0/5	0/5	0/7	0/7
Mesenterial lymph node	0/4	0/4	2/5	2/5

G = granulomas present, B = acid fast bacilli present.
Numerator indicates number of animals with positive findings, denominator indicates number of animals from which histological sections were examined.



Fig. 9. Detail from a subcutaneous aggregate of macrophages showing cluster of plasma cells. C3H mouse inoculated subcutaneously 30 weeks previously with 1.2×10^{6} *M. lepraemurium*. HE. × 765.

the lymph nodes, some of the nodules containing visible acid-fast bacilli.

Ten weeks after inoculation, scattered bacilli were found in the liver and spleen of C57/BL mice, while at this stage bacilli could not be demonstrated in the same organs of C3H animals. The bacilli that were present in the liver of C57/BL mice were found within small clusters of macrophages encircled by lymphocytes, indicating that they had given rise to a granulomatous reaction.

Thirty to thirty-three weeks after inoculation the liver was the only organ in which infection was regularly present in both strains (Table 1), but there was a marked strain difference as to what degree the liver was involved. In some C3H mice the whole organ was seething with foci, all densely filled with acid-fast bacilli, while in others only a few lesions were found. With the exception of one animal in which no bacilli were found in the liver some densely bacilli-laden cells could always be detected in this strain; characteristically such cells were never surrounded by lymphocytes. In the livers of C57/BL mice the number of lesions could be as high as in the moderately affected C3H animals, but the lesions always showed lymphocytic infiltration and contained far less bacilli than in the other strain. In fact, most of the granulomas did not contain any detectable acid-fast bacilli, and the total amount of bacilli appeared to be similar to that observed 23 weeks earlier, i.e. at 10 weeks after inoculation. All C3H mice examined showed extensive infiltration of the spleen with macrophages loaded with acid-fast material, while bacilli could be demonstrated in only 5 of the 7 C57/BL mice and in very small amounts. Spread of the infection to other sites was uncommon except in severely affected C3H mice. Furthermore a tendency towards more extensive spread was observed in the two C57/BL mice with reactivated infection. Spread of the infection to the thymus was more common in C57/BL than in C3H mice. The significance of this observation is not clear.

Histological findings in superinfected animals. Examination of the lesions produced by superinfection in C57/BL animals at 13 weeks showed that the inner bacilli-containing zone was proportionately smaller than the outer reactive zone, compared with the primary lesions. Besides, more fibrous tissue was separating the two zones, indicating greater fibroblastic activity. The outer reactive zone was composed mainly of macrophages, lymphocytes, and epithelioid cells, but a comparison of the proportion in which the respective cell types are present in the two types of lesions did not seem feasible. In C3H mice no signs of host reaction was seen when the lesions were examined after 30 weeks of infection.

DISCUSSION

The macroscopic appearance of lepromas developing after subcutaneous inoculation of mice with MLM has been extensively studied by *Kawaguchi* (1959) and formed the basis for his concept of a benign and a malignant form of murine leprosy. He introduced what he called the "leproma index", which to some degree seemed to correlate with the weight of the lepromas, and his comparison of the course of the infection in various inbred strains of mice is based mainly on this parameter. In the present study we have made similar comparison based on measurements of the diameter of the lesions. Since the lepromas in C3H are not well-defined they are probably as difficult to weigh accurately as they are to measure by size. Therefore, a comparison of the course of the infection in different mouse strains should be based on additional criteria.

Based on histological examination of the lesions we consider the infection to exhibit three stages (*Closs & Haugen* 1975)

1) An acute inflammation that starts within hours after inoculation and lasts less than one week, 2) a compatible stage when the bacilli multiply within mononuclear phagocytes, and 3) a granulomatous stage starting about 4 weeks after inoculation in C57/BL mice. The third stage does not develop in C3H mice and therefore the bacilli continue to multiply in this strain. When the granulomatous stage begins, the spread of the infection apparently stops and the amount of acid fast material ceases to increase. When the lesion becomes palpable in C57/BL mice it consists mainly of reactive cells, while in C3H mice, it consists mainly of a large uniform aggregate of macrophages completely filled with bacilli. In the first instance the palpable lesion thus is the result of a host reaction, while in the second it is due to multiplication of bacilli and therefore to lack of host reaction.

According to *Kawaguchi* (1959) regression of the infection in the benign form does not occur until 18–24 weeks after inoculation. In our opinion, regression of the infection, in terms of bacterial multiplication, starts as early as 4 weeks, i.e. with the onset of the granulomatous reaction. This early stage *Kawaguchi* (1959) regards as the progressive stage of the infection because the lesions then greatly increase in size. Thus, conclusions based merely on "clinical observations" appear to be misleading when they are not correlated with histological studies.

The demonstration of bacilli in the local lymph nodes shortly after inoculation shows that early spread of the infection took place in both strains. Thus, we have not succeeded in producing a strictly localized experimental infection. However proliferation of bacilli outside the local lesion appears to be very restricted in C57/BL mice since they exhibited no visible increase in the amount of bacilli in the liver in the period from 10 to 33 weeks after inoculation. In the C57/BL strain there also appears to be minimal secondary spread of bacilli from the local focus during the last part of the observation period. This indicates altogether that the host reaction in this strain is strong enough to suppress the infection. In the C3H strain the early spread of bacilli may be less than in C57/BL mice, but the secondary spread is profuse and the proliferation of bacilli apparently unrestricted.

An important question is whether the macrophages in C57/BL mice acquire the ability to kill the MLM or not. The histological findings indicate that two "compartments" are formed in the granulomatous lesions of these animals. In the central compartment, lymphocytes are scarce or absent and the macrophages contain large numbers of bacilli which stain well and probably should be regarded as viable. In the outer compartment bacilli are scarce and mainly elongated irregularly stained forms are seen indicating that these bacilli are dead (*Rees & Valentine* 1964). Lymphocytes are numerous in this part of the granuloma and many of the mononuclear phagocytes have the morphological characteristics of epithelioid cells which may be regarded as immunological markers (*Elves*

& *Epstein* 1968) indicating a high degree of activation (*Papadimitriou* & *Spector* 1971). Moderate deposits of collagen and fibrous tissue seem to form a barrier interfering with the free exchange of material between the two compartments. These observations suggest that a different degree of macrophage activation exists in the two compartments. Whereas the host reaction may be strong enough to destroy the bacilli in the outer zone of the lesion, the architecture of the lesion seems to prevent an effective attack on the bacilli in the central parts of it. A reduction in the bacillary load of the central part of the lesion can probably only be achieved through abscess formation, ulceration of the skin, and discharge of infectious material to the surface.

The present observations on superinfection show that when C57/BL mice are injected twice with an interval of 1 ½ weeks, the two lesions will simultaneously reach the granulomatous stage, i.e. the second inoculum gives rise to a reactive nodule more quickly than the primary inoculum. This implies that the initiation of the local granulomatous reaction depends on the development of a systemic immune response and not on local factors produced at the site of infection. After superinfection the subcutaneous lesion becomes smaller than when the same dose of bacilli are injected into a previously non-infected animal. Histologically the lesions arising after superinfection contain less bacilli but more reactive cells. This indicates that in the C57/BL strain infection with MLM produces some degree of increased host resistance against a subsequent challenge with this organism. In the C3H strain the superinfection lepromas also tended to be smaller than those of primary infection, but the reason for this remains obscure since no signs of an increased host response were detected histologically.

In some of the animals of the C57/BL strain the local infection suddenly started to progress after several weeks. Obviously this was associated with a decrease in cell mediated immunity since the histological appearance of the lesions of these animals converted to the "C3H type": bacilli-containing macrophages were accumulating in the tissues without any reactive cells surrounding them. The mechanisms behind such a shift in reactivity are at present unknown but it may represent a parallel to the downgrading of host resistance observed in untreated cases of human leprosy (*Ridley* & *Jopling* 1966).

In the lepromatous form of human leprosy antibodies against a variety of mycobacterial antigens are found in the patient's serum (*Rees et al.* 1965, *Myrvang et al.* 1974). We have not yet studied antibody production in the present model. However, the demonstration of plasma cells in the lesions of C3H mice shows that in this strain immunoglobulin is produced at the site of infection; presumably this represents local antibody production against mycobacterial antigens.

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ORAL GIANT CELL GRANULOMAS

An Ultrastructural Study of the Vessels

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Andersen, L., Fejerskov O & Theilade J Oral giant cell granulomas. An ultrastructural study of the vessels. Acta path. microbiol. scand. Sect. A, 83 69–76 1975

Eight peripheral and 2 central oral giant cell granulomas have been studied in the electron microscope in order to examine the structure and nature of the vessels. The vascular wall was composed of a continuous layer of endothelial cells resting on a basal lamina, which was frequently multilayered. The lamina also surrounded scattered subjacent pericytes. The endothelial cells overlapped slightly so as to form narrow intercellular spaces. Based on these observations the vessels are supposed to be postcapillary venules. Dark cells exhibiting degenerative features were encountered in the endothelial lining of most vessels. Small gaps and larger defects in the vascular lining allowed direct continuity between the lumen and the perivascular tissue. It is suggested that the extreme extravasation of red blood cells frequently observed in oral giant cell granulomas may be mediated through this incomplete lining.

Key words Giant cell granulomas oral vessels; ultrastructure.

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The oral giant cell granulomas (peripheral and central) have been the subject of many investigations during the last decade. Their biological behaviour and histopathology makes it reasonable to consider both types of lesions as manifestations of the same pathological condition (*Sapp* 1972, *Andersen et al.* 1973a) Histologically these highly vascular growths are among other things characterized by containing numerous extravasated erythrocytes and fibrin pools in the vicinity of the blood vessels and the giant cells (*Kramer* 1962 *Andersen et al* 1973a) This finding suggests that there may be defects in the vascular permeability and/or vascular leakages. Little attention has, however been paid to the structure and nature of the vessels in the granulomas (*Adkins & Romaniuk* 1971 *Sosolne* 1972)

Therefore, the present investigation was undertaken in order to study the ultrastructure of the vessels in the oral giant cell granulomas.

MATERIAL AND METHODS

The material comprised 8 peripheral and 2 central giant cell granulomas. The tissue was prefixed in a 6.25 per cent glutaraldehyde solution or in a combined paraformaldehyde-glutaraldehyde solution (*Karnovsky* 1965) and postfixed in 2 per cent osmium tetroxide before dehydration and embedding in Epon or Vestopal. Semithin sections were stained with toluidine-blue or paraphenylene-diamine (*Estable-Puig et al.* 1965) From



Fig 1 Light micrograph showing part of peripheral oral giant cell granuloma containing a vessel (V) The vascular lining is composed of flattened endothelial cells. Note the numerous extravasated erythrocytes. 1 µm section stained with toluidine-blue, × 330.

Fig 2 Vessels in central oral giant cell granuloma. The endothelial cells protrude into the lumen, when cut through the plane of the nucleus (arrow-heads) Giant cell (G) in close contact to the vessel wall. 1 µm section stained with toluidine-blue, × 330.

these sections, which were used in a previous study (*Andersen et al.* 1973b) areas were selected for the ultrastructural study Ultrathin sections were stained with uranyl magnesium acetate and lead citrate (*Frasc & Parks* 1965 *Reynolds* 1963) before examination in a Philips EM 200 electron microscope.

RESULTS

In the light microscope vessels of varying diameter were found throughout the giant cell granulomas. The vessels in the peripheral granulomas were similar to those of the central ones. The vascular lining generally consisted of flattened endothelial cells (Figs. 1 and 2) the nuclei of the individual cells protruding into the lumen. The vessels were surrounded by a varying number of giant cells, which occasionally formed part of the vascular lining (Fig 2) Some were lying free in the lumen.

At the ultrastructural level the vessels were seen to be composed of a continuous layer of endothelial cells, which rested on a basal lamina (Fig 3) One or more pericytes were surrounded by this lamina, thus contributing to the vessel wall. At several locations the lamina densa was multilayered.

Fig 3 Low magnification electron micrograph demonstrating a typical vessel. Numerous cytoplasmic projections are seen at the luminal surface of the endothelial cells. Multilayered basal lamina (arrow heads) and pericytes (P) × 3,000

Fig 4 Part of intercellular relation seen in Fig. 3 at higher magnification. Adjoining endothelial cells overlap forming narrow intercellular spaces. × 20,000.

P

P

3





Fig 5 Fenestrated endothelium (arrows) of a vessel. Pinocytic vesicles (arrow-head) × 29,000.

Fig 6 A detail view of the vessel seen in the inset. The endothelial cell shows a highly electron-dense cytoplasm and nucleoplasm. Both endoplasmic reticulum and nuclear envelope are dilated. Vascular lumen (L) × 14,500 inset × [illegible],500.

The general cytology of the endothelial cells was similar to what has previously been described. Thus, numerous ribosomes and fine filaments were found in the cytoplasm, and mitochondria and endoplasmic reticulum were present in moderate number. The Golgi-complex was situated close to the nucleus and a few lysosome-like structures were observed. Pinocytic vesicles were numerous along the cell membrane both facing the lumen and the surrounding tissue. From the luminal surface of the endothelial cell several cytoplasmic projections devoid of organelles were frequently found (Fig 9)



Fig 7. Part of a vessel demonstrating a defect in the endothelial lining. The lamina densa (arrows) appears intact. Endothelial cell (E) partly detached from the lamina. Stromal cell (S) showing degenerative features, × 14,500.

Fig 8. Part of vessel wall demonstrating gaps filled in with fibrin clots (F). Stromal cell (S) × 14,000.

The endothelial cells overlapped slightly so as to form narrow intercellular spaces (Fig 4); an occasional tight junction was located close to the lumen. Some vessels exhibited small fenestrae closed by a diaphragm, which appeared to be continuous on either side with the endothelial cell membrane (Fig 5).

The pericytes were highly branched in some areas making membrane contact to the endothelial cells. The cytology of these cells was similar to that of the endothelial cells, except for fewer mitochondria and cisternae of endoplasmic reticulum. Pinocytic vesicles could be seen mainly along the cell membrane opposite the vascular lumen.

Most vessels demonstrated one or more endothelial cells with an appearance different from that described above. The cytoplasm was very electron-dense, and the mitochondria were swollen with only a few cristae

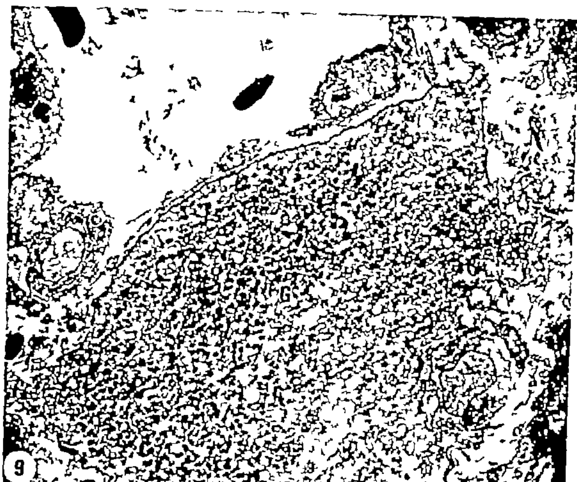


Fig 9 Giant cell (G) containing numerous vesicles and vacuoles close to the vessel wall, × 3,500.

evident (Fig 6) The nuclei were irregular in outline containing a very electron-dense nucleoplasm. The cisternae of the nuclear envelope and endoplasmic reticulum were dilated.

On occasion small gaps or larger defects were seen in the endothelial lining, allowing direct continuity between the vascular lumen and the perivascular connective tissue (Fig 7) although a lamina densa like structu might be interposed. Pericyte elements never observed in these areas of the va wall. The gaps often contained an ele dense fibrin clot extending into the ing tissue as well as into the lumen (Fi The endothelial cells t the def regularly showed a par the lamina densa, thu into lumen.

When giant cells showed a close relationship to the vessels, flattened endothelial cells were regularly observed between the giant cell and the lumen (Fig 9) In these areas the lamina densa was frequently absent but no membrane contact between the cells was observed. In some areas the endothelium was lacking, and remnants of endothelial cells and aggregations of a floc electron-dense material partly ant cells. hese cells containing les and oles thus formed lar

juxta vascular tissue loos d collagen fib fied filaments, cumulations frequently

DISCUSSION

A definitive classification of blood vessel types, such as that of *Rhodin* (1968) is very difficult to make in the present study on oral giant cell granulomas, and the exact relationship of the vessels to the vascular system as a whole cannot be determined. As the vessels were following a very irregular course it was impossible to decide the exact diameter of the vascular lumen because of difficulties in ascertaining whether the vessels had been cross-sectioned or cut more or less obliquely. Furthermore the vascular caliber in pathological tissue may be different from that observed in normal tissue, and as the methods of fixation and processing may influence the dimensions, such measurements will not be comparable. Consequently the structure of the vascular wall is the only reliable criterion which can be used for classification of the vessels in the present study.

The general structure of the vascular lining observed in the oral giant cell granulomas studied indicates that they may be classified as capillaries and/or postcapillary venules (*Majno* 1965, *Rhodin* 1968). The presence of pericytes excludes them from being lymphatic capillaries (*Majno* 1965). Vascular structures resembling lymphatic vessels have recently been described by *Sokolne* (1972) in a study on three peripheral giant cell granulomas, but these observations are based mainly on light microscopical examinations.

According to the degree of completeness of the endothelial layer *Majno* (1965) has classified capillaries into three types: continuous, fenestrated, and discontinuous capillaries. However a sharp distinction between the first two is probably not possible, but sinusoids can be distinguished as they are primarily found between the discontinuous capillaries. Sinusoids composed of loosely arranged stromal cells have been reported in giant cell tumours of bone (*Hanaoka et al.* 1970) which histologically show a certain similarity to the giant cell granulomas of the oral cavity (*Lucas* 1972). Vascular channels of this type were not identified in the present oral giant cell granulomas. The finding of sinusoids in these bone tumours may however be explained by the fact that this type of vessel is normally observed in the red marrow of long bones (*Majno* 1965).

The presence of numerous pinocytic vesicles and the positive reaction of adenosine triphosphatase in the endothelial cells demonstrated in a previous study (*Andersen et al.* 1973b) supports the view that the vessels of the oral giant cell granulomas are not typical fenestrated capillaries (*Marchesi & Barnett* 1963). Vascular gaps and leakages and a multilayered basal lamina have been reported as characteristic for regenerating capillaries in granulation tissue produced by mechanical or thermal injury in different types of tissues (*Schoefl* 1963, *Cotran* 1965, *McKinney & Panner* 1972, and *Vracko & Benditt* 1972). The vessels of the oral giant cell granulomas shows some features similar to these of regenerating capillaries, but the amount of pinocytic vesicles observed in the present study are only rarely observed in regenerating capillaries (*Schoefl* 1963).

However, the general structure of the vascular wall including the multiplications of the lamina indicates that they are postcapillary venules (*Rhodin* 1968, *Majno* 1965). The postcapillary venules have commonly been included in the term "capillary", but as the venules behave differently from capillaries under normal and abnormal conditions *Majno* (1965) has stressed that a distinction has to be made. Thus the venules are more susceptible to different types of injury and are a likely site of haemorrhage and diapedesis. This could explain the content of numerous extravasated erythrocytes and pools of fibrin in the giant cell granulomas (*Kramer* 1962, *Andersen et al.* 1973b). Further the present investigation has shown gaps and leakages in the vascular lining, where erythrocytes are able to squeeze through because of their extreme plasticity. The electron-dense endothelial cells have been interpreted as degenerative changes in the vascular lining, which may facilitate haemorrhage. Similar endothelial changes have been observed in

liver tissue with experimentally induced extrahepatic biliary obstruction (*Steiner et al.* 1962)

In conclusion the described vascular changes can explain the findings of numerous erythrocytes lying free in the giant cell granulomas, but it is surprising that not more haemosiderin-containing macrophages are encountered in the biopsies. It can not be excluded that the extravasation of erythrocytes are accentuated by mechanical damage of the tissue in situ or during the surgical procedure.

The authors are indebted to Dr *Christophe Squier* Department of Oral Pathology Dental School, University of London, The London Hospital Medical College Turner Street, London E 1 for helpfull correction of the manuscript.

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A NEW METHOD FOR THE EMBEDDING OF SMALL ANIMALS AND ORGANS IN PLASTIC

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Larsson, Sven-Åke. A new method for the embedding of small animals and organs in plastic. Acta path. microbiol. scand. Sect. A, 83: 77–79 1975

A new method for embedding of small animals and animal organs in plastic is described. After the material has been fixed in solutions preserving its colour and dehydrated gradually using an aqueous solution of glycerol and potassium acetate it is embedded in methylmetacrylate. The method is described in detail and involves moulding at –5 C.

Key words Plastic organ preservation embedding method.

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Nowadays the preservation of small animals, healthy and diseased animal organs and parts of organs, is mainly done by embedding them in plastic. When the department of pathology at the National Veterinary Institute changed from wet preparations to this method, the American polyester Castolite was initially used as described by *Notini & von Wettstein* (1961). However the transparency of the preparations was not good and optical phenomena sometimes occurred. In 1969 the department changed to a German glass-clear monomer methylmetacrylate called Plexit 55®* which proved to be very suitable despite the disadvantages. The moulding process is somewhat complex and the plastic contains volatile substances and solvents which are poisonous to inhale.

Since the department started to use this plastic, a method has been developed for preservation, which seems to have many advantages over those previously described in the available literature (Figs. 1–4). The method is here described in four sections fixation, dehydration, embedding in plastic, and aftertreatment.

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Fixation

When internal organs or parts of organs such as liver kidney heart etc are fixed it is particularly important that they are placed in the fixing liquid as rapidly as possible before drying out of surfaces occurs.

One of the following methods may be used for fixation

A. The organs are placed in a 10 per cent aqueous solution of formaldehyde for approximately 14 days. When fixed in formalin the organs lose their natural colour and become greyish. They can regain their natural colour in the following modification of Romhanyi's (1956) solution.

Formaldehyde 37 per cent	150 g
Nicotine	10 g
Sodium dithionite	30 g
Distilled water	1000 ml

After a few hours the solution has reacted with the blood pigment in the organs and the organs resume most of their original appearance

B. The organs can be fixed for –3 weeks in a solution recommended by Jores (1913)

liver tissue with experimentally induced extrahepatic biliary obstruction (*Steiner et al* 1962)

In conclusion the described vascular changes can explain the findings of numerous erythrocytes lying free in the giant cell granulomas, but it is surprising that not more haemosiderin-containing macrophages are encountered in the biopsies. It can not be excluded that the extravasation of erythrocytes are accentuated by mechanical damage of the tissue in situ or during the surgical procedure.

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tically on a stand. On the same stand, two Philips TLAD 15W/05 fluorescent lamps are placed above the plate and two below. These fluorescent lamps are movable both vertically and horizontally according to the height and breadth of the mould used. Polythene is a suitable material for the moulds as it has a good resistance to the unpolymerized plastic and can easily be detached from the polymerized plastic. To enable the plastic to be illuminated both from above and below the mould consists of a frame which is placed on a glass plate acting as a bottom. To ensure that the plastic can be freed from the glass plate, sheet of wet cellophane is placed on the glass and smoothed out with a rubber roller. The edges are then folded up round the sides of the frame. A glass plate with a hole, diameter approximately 10 mm, is used as a lid on top of the mould. A contact thermometer is placed in the hole to register the temperature rise developed in the plastic during polymerization. The thermometer which is set at 30° C and connected to an electronic relay breaks the current to the lamps when the temperature in the mould becomes too high. Thus the polymerization can occur continously without attention. Despite the low temperature registered in the mould during polymerization, the objects in the plastic are sometimes subjected to too high a temperature manifesting itself by discoloration and precipitates in the plastic. This can be avoided by lowering the moulding temperature to below 0° C. The apparatus with the fluorescent lamps previously described is placed in a freezer the thermostat being set at –5 C. This method of embedding has several advantages. The organs retain their natural colour better and the fixing liquid still present in the organs becomes so viscid that it is usually not pressed out of the organs by the compression arising from polymerization.

The radiation apparatus described above is used for the moulding of the bottom layer before the preparations to be embedded are placed in the mould, and for final polymerization when little heat is generated. Because of the possibility of poisoning, all work by which the plastic may become olatile must be carried out in a fume cupboard. The plastic also attacks the skin, and hands must thus be protected (special chemical gloves are most suitable as the gases penetrate rubber gloves).

The Embedding P oced re

The best result is obtained if the embedding is made in the following steps.

1. After dehydration, dry the specimen thoroughly and place it in a dish with plastic without catalyst.

2. Place the dish in an evacuating glass desiccator. The air in the preparation is then removed and replaced by plastic.

3. Put a 10 mm deep layer of plastic with 2 per cent catalyst in a frame placed on a glass plate and let it polymerize in the radiation apparatus at maximum 30 C. It takes approximately 1 hour for it to solidify to a jelly-like consistency.

4. Press down the specimen in this plastic in such a way that no air bubbles remain under the preparation. This can easily be checked through the glass plate at the bottom of the mould.

5. Plastic with catalyst is added to a level of 15–20 mm above the specimen. A specimen with a volume of 0.5 litre requires a catalyst of 2–3 per cent and for 1 litre or more 0.5–1 per cent. To remove possible contaminants and air bubbles before the plastic polymerizes, a hard paintbrush and a dental probe sharpened to a point are used. It is important that air bubbles immediately adjacent to the specimens are removed. The remaining air bubbles which lie in the plastic will disappear during polymerization.

6. Put the mould in the radiation apparatus placed in a freezer at –5 C. After 2 days the plastic has attained a solid consistency.

7. The final polymerization can be carried out at room temperature in the radiation apparatus described in point 3. The result is a hard, glass-like surface.

Aftertreatment

When the plastic block has been removed from the mould, it must be treated so as to obtain a glass-like finish (Figs. 1–4). This type of plastic is not suitable for polishing on a normal abrasive belt as the heat generated due to contact with the belt causes small cracks in the surface of the plastic. Flat face milling is therefore recommended. Another advantage of flat face milling is that plane surfaces and right angles can be obtained. Before polishing, which is carried out on a normal open polishing wheel, the plastic block must be finely ground with a waterproof paper. To prevent scratching, small "feet may be attached to it. These can be made of a 6 mm plexiglass rod, 1–2 mm high, which are glued on and thereafter polished with a polishing wheel.

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THE HISTOGENETIC-EMBRYOLOGIC BASIS FOR REAPPEARANCE OF ALPHA-FETOPROTEIN IN ENDODERMAL SINUS TUMORS (YOLK SAC TUMORS) AND TERATOMAS

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Teilum, G., Albrechtsen, R. & Norgaard Pedersen, B. The histogenetic-embryologic basis for reappearance of alpha-fetoprotein in endodermal sinus tumors (yolk sac tumors) and teratomas. Acta path. microbiol. scand. Sect. A, 83 80–86, 1975

The mechanism of neosynthesis of the human tumor-associated fetal antigen alpha-fetoprotein (AFP) in a variable percentage of patients with testicular, ovarian and extragonadal germ cell tumors has generally been considered unknown or beyond any simple explanation. Of decisive importance is the cellular basis for AFP production 1. in ontogenesis and 2. in malignancy as dependent on an exact tumor histogenesis. Based on (1) the histogenetic embryologic classification of germ cell tumors and the concept of yolk sac tumor (or endodermal sinus tumor), (2) the available clinical and experimental observations, and (3) the immunofluorescent localization of AFP in the endodermal sinus tumor of the human testis, it is concluded that AFP synthesis in these neoplasms is explained by the fact that they contain yolk sac endoderm, which produce AFP analogous with the physiological AFP synthesis by the fetal yolk sac in early embryogenesis.

Key words: Alpha-fetoprotein; endodermal sinus tumors; teratomas.

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Alpha-fetoprotein (AFP) was first demonstrated by *Bergstrand & Czar* in 1956 in serum from human fetus. It is an alpha-1-globulin, and in the early embryo it is probably the dominant serum protein being replaced later by albumin, which it resembles in physico-chemical properties. In the human embryo it is produced first by the yolk sac and later by the liver. A maximal concentration of AFP is found at about 12 to 15 weeks of gestation, after which it declines slowly and within a few weeks after birth the serum levels fall rapidly to the adult level, which is so low that it is difficult to detect even with the more sensitive assays.

The observation that fetal antigens could be released into the body fluids from human tumors has opened a new field within the functional pathology. The AFP test already

has a clinical role and offers new insight into the origin, specific nature and detection of a tumor and also serves as an indicator of recurrence and as a monitor of therapeutic progress.

AFP and Germ Cell Tumors

Abelev et al. 1967 reported that AFP appears not only in sera of patients with hepatocellular carcinoma, but also in many cases of testicular "teratoblastoma which exhibited some elements of embryonal carcinoma. The test was negative in other types of testicular tumors, including seminoma, chorionepithelioma and stromal tumors. *Masopust et al.* 1968 in an extensive analysis of sera from 248 patients, children and adults with various tumors, and from 494 patients with different non-neoplastic diseases, stated that AFP appears in two types of tumor only hepatocellular carcinoma and "malignant undifferentiated teratoma" In other types including nephroblastoma, sympathicoblastoma, chorionepithelioma and dysgerminoma, AFP was not detected in any patients. In a study of sera from 93 children bearing various malignant tumors *Mawas et al* 1969 found AFP positive in one testicular and four ovarian embryonal carcinoma"

Reports by these three groups (2, 15 16) and several other investigators show that germ cell tumors not only of the testis or ovary but also of various extragonadal sites may produce AFP Generally however the reported cases have been described in indefinite terms, such as teratoblastoma" "teratocarcinoma" malignant undifferentiated teratoma" or "embryonal carcinoma" without attempts to correlate a specific histology of the tumors to the presence or absence of AFP It also remained unexplained why a number of sera from patients with such tumors were AFP negative. Furthermore some confusion has existed regarding the various cellular elements of the tumor and the degree of embryological differentiation related to AFP synthesis.

Neosynthesis of AFP

Most investigators have stated that the basis for the reappearance of the fetal protein in a variable percentage of patients with testicular carcinomas is unknown (*Grooser & Rogers* 1973 *Lehmann & Lehmann* 1974) or "beyond any simple explanation" (*Masopust et al* 1968) which would not exclude a "heterotopic synthesis" by an embryonal tumor "even when the neoplasm exhibits no histologic similarity to the tissue producing AFP physiologically" The theory currently accepted for the AFP reappearance in serum of certain patients with these testicular and ovarian tumors is that genes which code for the production of AFP are repressed late in fetal life and become derepressed when malignancy supervenes. (*Abelev* 1968, *Smith & O Neill* 1971 *Mernn et al.* 1973)

Tumor extract studies have provided evidence that AFP synthesis takes place in the tumor itself (16 23 25)

The purpose of this paper is to discuss the mechanism of neosynthesis of AFP in patients with testicular ovarian, and extragonadal germ cell tumors.

AFP Synthesis and Histogenetic Classification of Yolk Sac Tumors

Of decisive importance is the cellular basis of AFP production 1 in ontogenesis and 2 in malignancy as dependent on a precise tumor histogenesis. *A priori* AFP should be expected to be produced just in tumors containing the analogues of cells responsible for AFP synthesis in ontogenesis. After the site of synthesis was demonstrated to reside not only in the embryonic hepatocytes, but also in the human yolk sac at early embryogenesis (*Gitlin & Perricelli* 1970) new attention has been focused consequently on the endodermal sinus tumor (or yolk sac tumor) which was recognized and classified on a histogenetic embryologic basis by *Teilum* in 1939 and which is now generally accepted as a distinct and specific entity While the previous classifications of germ cell tumors are useless as regards to the cellular basis for AFP produc

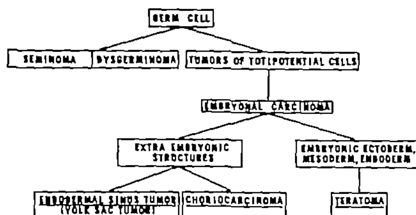


Fig 1 Histogenesis and interrelationship of ovarian and testicular tumors of germ cell origin. In this classification the term "embryonal carcinoma" is restricted to tumors composed of undifferentiated totipotential embryonal cells, representing the undifferentiated form of the extra-embryonic as well as embryonic tumor types (From *Teilum* 1965)

tion, the histogenetic classification advocated by Teilum 1959 1965 and 1971 has been the key to the solution of this problem. According to Teilums classification (Fig 1) the embryologically differentiated forms from the multipotent stem cell of embryonal carcinoma are subdivided into the following three types 1 Endodermal sinus tumor (yolk sac tumor) 2. Choriocarcinoma. 3 Teratoma. Any combination of these three types and of these and the embryologically undifferentiated embryonal carcinoma may occur (Fig 2)

With this classification in mind there are already several reports of serum AFP in patients with ovarian and testicular tumors diagnosed as specific endodermal sinus tumors. Two cases of endodermal sinus tumor in the ovary were described by *Ballas* in 1972 and *Wilkinson et al.* in 1973 *Tsuchida et al* in 1973 reported 3 cases of malignant teratoma in children (two originating in the testis and the third in the presacral region) Sequential evaluations revealed that the production rate of AFP closely paralleled the amount of the visible malignant tumor tissue which fluctuated in the course of the diseases. After the microscopic sections were carefully reviewed it was concluded that the cases are properly classified as yolk sac tumor or endodermal sinus tumor

The pure form of endodermal sinus tumor is most often found in the ovary of young women and children, and in the testis of infants (Fig 3) but in the adult testis it is relatively rare (Fig 4) It is, however well established that some of the histological patterns of "embryonal carcinoma" and "teratocarcinoma" of the adult testis actually represent extraembryonic vitelline structures (21) accounting for the AFP production (16) (Fig 5)

In such cases the vitelline component may not constitute the bulk of the tumor and progression of the disease may be caused by other components not producing AFP (7 9) (cf. also the ovarian tumor Fig 2A and B)

In an immunofluorescent study of AFP in transplantable murine teratocarcinoma *Engelhardt et al* 1973 found AFP-specific fluorescence in epithelium of endodermal origin lining cysts and tubules. *Teilum et al.* 1974 by immunofluorescence-microscopy studied a typical and pure form of endodermal sinus tumor of the testis and demonstrated for the first time on human material that AFP is synthesized by cells of the yolk sac endoderm lining the endodermal sinuses, thus accounting for the increased AFP concentration in both serum and tumor homogenate. Furthermore, it was shown that the intra- and extra-



Fig. 2 Combined germ cell tumor of the ovary showing: A. Endodermal sinus tumor B. Choriocarcinoma. Alpha-fetoprotein 950 μg/L. Urinary chorionic gonadotrophin 87000 I.u./l (20-year-old patient) H.E., × 210. (Courtesy Dr *J. F. Thorberg*, Randers, Denmark)

Fig 3 Pure endodermal sinus tumor of the infant testis. Alpha-fetoprotein 2210 μg/L (21-month-old patient) H.E., × 84

Fig 4 Pure endodermal sinus tumor of the adult testis (23-year-old patient) H.E., × 150

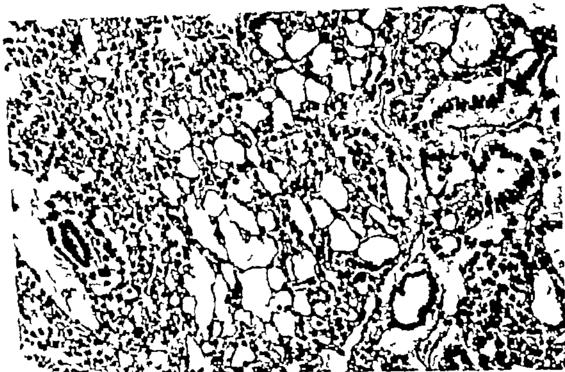


Fig. 5. Embryonal carcinoma of testis histologically showing a marked vitelline component. Alpha-feto-protein 4000 μg/L (32-year-old patient) H.E., × 210.

cellular PAS-positive hyaline globules, characteristically found in many endodermal sinus tumors, contained AFP

The histogenetic interpretation of these germ cell tumors (Fig. 1) in accordance with the clinical and experimental observations would explain the neosynthesis of AFP as being dependent on the presence of a significant vitelline component producing AFP analogous with the AFP synthesis of the fetal yolk sac in early embryogenesis.

DISCUSSION AND CONCLUSIONS

It is concluded that the histogenetic classification of germ cell tumors (20 21 22) offers a reasonable explanation of the neo-secretion of AFP in endodermal sinus tumors and many cases of "teratocarcinoma" The same explanation based on the histogenetic interpretation has recently been given by *Abelev et al. Tsuchida et al.* and has been suggested in several case reports (5 23) and also assented to by *Masopust* (14)

According to the histogenetic system it is not the teratoid component (undifferentiated or differentiated) or the undifferentiated embryonal carcinoma component of a teratocarcinoma which is responsible for the AFP synthesis of the tumor It has also been demonstrated that the pure, embryologically highly differentiated endodermal sinus tumors are among the most active AFP producers. The commonly presented assumption (12) that AFP synthesis is directly related to the undifferentiated or embryonal carcinoma cells of the teratoma is untenable. It should be realized that the yolk sac elements embryologically represent a higher degree of differentiation than the embryonal carcinoma cells. The decisive point is the direction of differentiation from the multipotential embryonal carcinoma cell into the extraembryonic yolk sac endoderm. However undifferentiated teratomas and embryonal carcinoma

may have contained multipotential cells which focally have differentiated to yolk sac endoderm accounting for the AFP secretion.

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SUBCLINICAL ELASTOFIBROMAS IN THE SCAPULAR REGION IN AN AUTOPSY SERIES

Additional Notes on the Aetiology and Pathogenesis of Elastofibroma Pseudoneoplasm

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Järvi, Osmo H. & Länsimies, P Hillevi. Subclinical elastofibromas in the scapular region in an autopsy series. Additional notes on the aetiology and pathogenesis of elastofibroma pseudoneoplasm. Acta path. microbiol. scand. Sect. A, 83 87–108, 1975

In a series of 235 autopsies, changes in the subscapular thoracic fascia similar to elastofibroma dorsi (*Järvi & Saxén* 1959 et al. 1969) were found in 39 cases, all at least 58 years old. In people over 55 years, the frequency was 24.4 per cent in females and 11.2 per cent in males. In addition to hypertrophy and secondary degeneration of elastic fibres, necrosis of collagenous- adipose- muscular and nervous tissue, as well as formation of clefts, cysts and bursae was found in 85 per cent of cases presenting elastic changes—both in connection with them and outside the degenerated areas—as well as in 39 per cent of cases where no elastic degeneration occurred. Other changes included extensive scarring of the tissue, followed by reduction of fat and, more seldom, oedema and inflammatory infiltration. Breaks in the elastic cage, necrosis and fibrosis of arterial walls were found in 44 per cent of cases of elastic degeneration and in 14 per cent of cases without degeneration. In veins, more extensive wall fibrosis occurred, leading to necrosis in cases of elastic degeneration the adventitial elastic network was also involved. Venous changes were found in 90 per cent of the cases of elastic degeneration and in 50 per cent of cases without degeneration. Direct mechanical stress on elastic tissue may be an important cause of hypertrophy and secondary degeneration of elastic fibres, and also of diffuse increase of collagenous tissue. On the other hand, nutritional deficiency due to failing resistance of the vascular system against friction of the scapula and streching movements of the upper extremities may play a main part in necrotic tissue changes.

Key words Elastofibromas scapular region autopsy series.

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At the twelfth Congress of Scandinavian Pathologists in 1959 *Järvi & Saxén* reported *four cases of* tumours in the subcapsular region which contained besides *collagen and* fat, a large amount of hypertrophic elastic fibres. The fibres were partly segmented and fragmented to form globules and debris. The authors called the tumour elastofibroma (dorsi) and suggested that friction of the apex of the scapula over the thoracic cage could contribute to the development of the tumour In a more extensive study by light and electron microscopy *Järvi et al* (1969) showed that the hypertrophy of elastic fibres was in fact mainly a proliferation of specific elastic fibrils around the original elastic fibres which could often be identified as a central

cord within the hypertrophied elastic mass. Breaks in this central cord could also be demonstrated and were thought to be the primary cause of fragmentation of the swollen fibres into globules. The latter study also supported the concept of elastofibroma as a pseudotumour resulting from mechanical overstrain. It was based on 15 cases seen personally by the authors and four specimens sent by pathologists from abroad. An additional 30 cases were known to the authors from the literature or by personal communication. Two of the published tumours occurred outside the subcapsular region, one on the left greater trochanter (*Barr* 1966) and the other on the ischial tuberosity (*Hausman & Smith* 1968)

Since the paper by *Jarvi et al* appeared in 1969 specimens from a further ten patients have been examined by ourselves or sent to this laboratory by other pathologists in Finland and a further two tumours removed from the opposite side in patients No 6 and 11 described in the paper mentioned above (Table 1) from whom a tumour had already been removed. In addition, a Japanese study by *Enjoji & Kikuchi* (1968) had been overlooked these authors described a case seen by themselves, namely a 71 year-old woman with bilateral tumours and a 10-year history and two cases found by other Japanese pathologists (a 73-year-old Japanese woman [with] a tumour on the left side and a middle [ag]ed Caucasian woman) *Gonzales-Crussi et al.* reported in 1969 a left-sided tumour in a 74-year-old woman *Freilich & Bendix* (1969) observed a right-sided tumour in a 73-year-old man (taylor) *Winkelmann & Sams* (1969) found a left-sided tumour in a 62 year-old man and *Cartanu & Eusebi* (1971) found a right-sided elastofibroma in a 57 year-old woman. Finally *Mirra* consulated one of the authors (O H J) concerning a tumour in the right deltoid region in a 53-year-old man which also proved to be elastofibroma (*Mirra et al.*) By now altogether 60 cases of elastofibroma are known to us.

The observations of changes similar to those in elastofibroma dorsi in the elastic tissue of Morton's intermetatarsal neuromas made by *Reed & Bliss* (1973) and also, independently by *Mirra* (9) indicate new achievements in studies of elastofibroma. In 15 cases of Morton's neuroma seen in our department, these changes were not demonstrated and thus the elastic alteration is not a regular characteristic of this disease.

In their study of 1969 *Järvi et al.* reported a routine autopsy case without any clinically identified tumour where microscopy revealed changes in the elastic fibres of the subcapsular thoracic fascia. This finding provided an incentive for a more extensive study of a Finnish post mortem series despite the negative experiences gained by *Barr* (1) in a similar investigation of 25 persons over the age of 65 The results have been presented at the Ninth International Congress of International Academy of Pathology 1972 (6) and shortly discussed in the paper by *Mirra et al.* (9)

MATERIAL AND METHODS

In 235 post mortem cases (133 females and 102 males) mainly middle aged or old people, the scapular region was dissected and the subcapsular thoracic fascia thoroughly examined. Several samples from the thoracic and subcapsular fascia were usually taken for histological examination The specimens were fixed in neutral formol and embedded in paraffin. Elastic tissue was stained with Verhoeff-van Gieson elastic and Gömöri's aldehyde fuchsin stain. Haematoxylin- an Gieson and AZAN (Heidenhain) trichrome stainings were used for connective tissue as well as Verhoeff's method.

RESULTS

The Frequency of Lesions

The findings in the study concerning frequency age, and sex distribution of elastic lesions are presented in tables 1 2 and 3 In 29 out of 119 females, and in 10 out of 89 males, over 55 changes identical to elastofibroma occurred in the elastic fibres in some area of the thoracic fascia. In most cases in which a lesion was found by microscopy it was also visible macroscopically as a thickened and indurated area in the thoracic fascia, often several centimeters in extent, the largest mea

TABLE 1 Occurrence of Elastic Degeneration, Tissue Necrosis or Bursae in the Scapular Thoracic Fascia in 235 Post Mortem Cases and Their Distribution in Age Groups Covering 5 Years Percentages in Italics

	25–	30–	35–	40–	45–	50–	55–	60–	65–	70–	75–	80–	85–	90–	Total
Females															
Elastic degeneration															
With tissue necrosis (or bursae)								3	3	1	6	8	1	2	24
Without tissue necrosis (or bursae)										3		2			5
Total								3	3	4	6	10	1	2	29
Percentage of female age groups								*14*	*21*	*22*	*21*	*40*	*20*	*67*	*21.8*
Without elastic degeneration															
Tissue necrosis (or bursae)		1		1		1		5	5	4	9	9		1	38
Percentage of female age groups		*50*		*20*		*25*		*23*	*42*	*22*	*32*	*36*	*0*	*33*	*27.1*
Smaller changes or normal		1	1	4	2	3	4	14	6	10	13	6	4		68
Total		2	1	5	2	4	4	19	11	14	22	15	4	1	104
Males															
Elastic degeneration															
With tissue necrosis (or bursae)							2			3		2		2	9
Without tissue necrosis (or bursae)									1						1
Total							2		1	3		2		2	10
Percentage of male age groups							*12*		*7*	*17*		*22*		*33*	*9.8*
Without elastic degeneration															
Tissue necrosis (or bursae)					1	1	4	5	7	7	7	6		2	40
Percentage of male age groups					*25*	*20*	*25*	*42*	*50*	*41*	*47*	*67*		*33*	*39.2*
Smaller changes or normal	1	1		2	3	4	10	7	6	7	8	1		2	32
Total	1	1		2	4	5	14	12	13	14	15	7		4	92
Total	1	3	1	7	6	9	20	34	28	35	43	34	5	9	235
Percentage of all cases	*0.4*	*1.3*	*0.4*	*3.0*	*2.6*	*3.8*	*8.5*	*14.5*	*11.9*	*14.9*	*18.3*	*14.5*	*2.1*	*3.8*	*100*



Fig 1 Macroscopic picture of a flat, slightly nodular thickening of thoracic fascia of 90-year-old male, which microscopically proved to be a subclinical elastofibroma. The dimensions of the thickening were 3 × 2.5 cm.

suring 3 × 2.5 cm (Fig 1) At times however the lesion occurred only as a streak in the fascia. In some cases the thickening consisted of a brittle, granular substance. Fascial thickenings without elastic regenerative and degenerative changes were, however also manifest, but showed other tissue alterations by microscopical examination (see page 20)

TABLES 2. *Cases of Elastic Degeneration of the Subcapsular Thoracic Fascia*

Sex	Age	Occupation	Elastic degen. right	Elastic degen. left
Female	60	Worker's wife	+	—
Female	61	Worker (retired)	+	+
Female	64	Cashier	+	—
Female	65	Restaur manageress (retired)	+	—
Female	66	House wife	+	+
Female	69	House wife	+	+
Female	70	Worker (retired)	+	—
Female	73	Farmer's wife	+	+
Female	73	House wife	+	—
Female	74	Teacher's widow	+	+
Female	75	Clerk's wife	+	—
Female	75	House wife	+	+
Female	77	Smith's widow	+	+
Female	78	Ironing woman (retired)	—	+
Female	79	Shop keeper's widow	+	+
Female	79	Worker's widow	+	+
Female	80	Caretakers's widow	—	+
Female	80	Charlady (retired)	+	—
Female	80	Worker's widow	+	+
Female	81	Factory hand (retired)	+	+
Female	82	Plater widow	+	+
Female	82	Joiner's widow	+	—
Female	82	Farmer's wife	—	+
Female	83	House maid (retired)	+	+
Female	84	Farmer's widow	+	+
Female	84	Joiner's widow	—	+
Female	87	Standkeeper (retired)	+	+
Female	90	Farmer' wife	—	+
Female	91	Shop keeper (retired)	—	+
Male	58	Turner	+	+
Male	59	Farmhand	+	—
Male	66	Sea captain (retired)	+	+
Male	70	Farmer	+	—
Male	71	Peddler (retired)	+	—
Male	74	Watchman (retired)	+	—
Male	82	Farmer (retired)	+	—
Male	83	Farmer (retired)	+	—
Mal	90	Farmhand (retired)	+	+
Male	92	Carpenter (retired)	—	+
Total	39	right side 14 bilateral 18, left side 7		

As clinical elastofibroma occurs mostly in elderly people, the age distribution in our series is interesting The youngest man in whom the elastic degeneration was found was 58 years old, the youngest woman being 60. The female preponderance was very marked. In females over 55 years, the frequency of degeneration was 24 4 per cent, against 11.2 per cent in males. Among 14 women and 13 men under 55 no positive cases were found. The frequency seemed to increase with age. The percentage of degenerative cases almost steadily increased in ten-year periods after the age of 55 years (among females the percentages in the period 55–64 yrs was 11.5, 65–74 yrs 21.9 75–84 yrs 30.2 and over 85, 37.5 among males the corresponding percentages were 7 1 12.9 8.3 and 33.3) On the consideration that the series of clinical

TABLE 3. *Distribution of Occupations According to Light- and Intellectual Work of Cases with Subclinical Elastofibroma and of Cases without Elastic Degeneration*

	Elastic degeneration		Without elastic degeneration		Total	
	cases	per cent	cases	per cent	cases	per cent
Females						
Heavy work	10	35	48	*46*	58	*44*
Light work	18	62	47	*45*	65	*49*
Intellectual work	1	3	9	*9*	10	*7*
Total	29	*100*	104	*100*	135	*100*
Males						
Heavy work	7	*70*	48	*51*	55	*54*
Light work	2	*20*	35	*38*	37	*36*
Intellectual work	1	*10*	9	*10*	10	*10*
Total	10	*100*	92	*100*	102	*100*

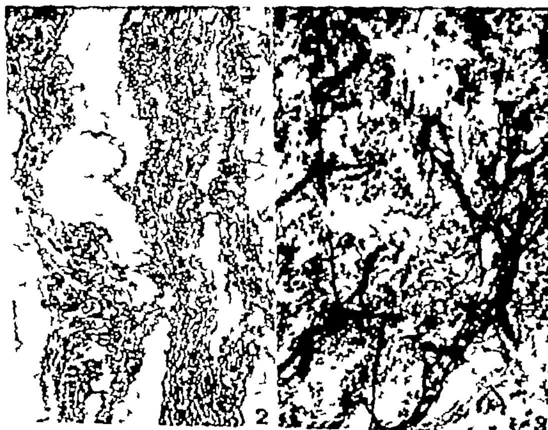


Fig. 2. Connective tissue bands with hypertrophic degenerated elastic fibres often broken into globules. Between the bands unchanged fat. Female, 73 years. (Verhoeff's stain. × 42)

Fig. 3. Compact fibrous tissue with advanced elastic degeneration. Some of the most coarse elastic fibres, however, unchanged. Female 83 years. (Verhoeff's stain. × 260)

elastofibroma included eight female patients under 55 years (and one described as "middle-aged") the youngest being 41 and two others (42 yrs and 44 yrs) with a history starting at 41 and one male of 45 years of age, it is to be expected that, in a larger series, the degeneration would occur in patients much younger than those included in the series now examined. The preponderance of women in the group of cases of degeneration is in agreement with the results obtained in the series of 38 females and 11 males with clinical, subscapular elastofibroma collected by Järvi et al. in 1969 and completed by us. Like the clinical lesion, the subclinical lesion was also slightly more common on the right side than on the left. In 18 cases it was bilateral, in 14 it was on the right side and in only seven on the left (Table 2)

Not much can be concluded from the occupation of the affected people (Table 2 and 3) All of the men but one, a sea captain, were labourers, two of whom did manually light work nineteen of the women were housewifes (three from upper social classes) six labourers, two shopkeepers, one restaurant manager and one cashier The series of cases without elastic degeneration included 9 men (10 per cent) and 9 women (9 per cent) who had done only intellectual work and 35 men (38 per cent) 47 women (including housewifes) (45 per cent) whose work was considered light. On the other hand, a closer inspection of the series of clinical cases shows that it includes several people who were occupied with light work one doctor three nurses, one shop manager one janitress and one foreman and, in some of these the lesion developed even at an early age. On the basis of the histories of some patients, the impression might be obtained that certain occupations which involve much manual work could favour the development of the disease, but on the other hand, many exceptions to this rule point rather to the importance of constitutional factors.

An age distribution similar to that in cases of subclinical elastofibromas was also found to apply to cases of tissue necrosis and burns formations not associated with elastic changes. The alterations, however were found in two patients less than 50 years old (Table 1) They occurred among females twice as often as elastic degeneration, but among males even five times as often as elastic degeneration. No explanation of this marked difference between the series can be given

As expected, any correlation between subclinical elastofibromas and the disease which caused death could not be found.

Histology of Subclinical Elastofibromas

In principle the microscopical changes were identical with the findings in cases of clinical elastofibromas. Streaks of collagenous connective tissue alternated with fat. The amount of collagen was increased, and the elastic fibres were thickened, usually to about 10 μ, although the largest ones measured were 17 μ in diameter and they were often broken

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Fig 4 Regenerative hypertrophy of elastic fibres in connective band. The original elastic fibre staining darker is covered by a lighter staining sheath. Both longitudinal and cross sections are visible. Almost in the centre of the picture, two original elastic fibres have a common sheet. No essential globular disintegration. Female, 79 years. (Gömöri's aldehyde-fuchsin stain. × 460)

Fig 5 Swollen elastic fibres in connective band with central cords and sheaths. Most of the sheaths show segmentation. Female 79 years. (Gömöri's aldehyde-fuchsin stain. × 400)

Fig 6 Swollen elastic fibres in the middle in unchanged adipose tissue. The thin original fibres are clearly discerned as well as the less intensively stained hypertrophic sheath around them. Same case as fig. 5 (Verhoeff stain. × 470)

Fig 7 Segmental and globular degeneration of hypertrophic elastic fibres in otherwise unchanged fatty tissue. Sometimes two parallel rows of globules are formed (e.g. upper right corner) Female 78 years. (Verhoeff's stain. × 430)

Fig 8 Elastic fibre with numerous short, knob-like, side branches "satellites" perhaps a regenerative phenomenon. Lower in the fibre, the central cord and sheath can be clearly discerned The fibre was lying in unchanged fat. Female 84 years (Verhoeff's stain. × 430)


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Fig 9 Well-defined necrosis in fibrous connective tissue in subscapular thoracal fascia. Fragments of collagenous and elastic fibres in the necrosis. Female, 73 years. Case without elastic degeneration. (Verhoeff's stain. × *105)*

Fig 10 Connective tissue necrosis from left subscapular region adjoining veins with extensive wall fibrosis and total disappearance of medial muscle cells. In the necrosis, collagenous bundles are swollen and broken and some fragments of elastic fibres occur but no cells or nuclei are preceptible. *Male, 71 years, with elastic degeneration* on the right side. (Verhoeff's stain. × 105)

Fig 11 Collagenous whirl probably caused by rupture of the bundle. Fine elastic fibres in the bundle. Male, 65 years, without elastic degeneration. (Verhoeff's stain. × 260)

into globules (Fig 2 3 24 33) In the enlarged elastic fibres the original fibre was often discerned as a central cord owing to its more intensive staining reaction with elastic stain (Figs. 4–6 8) As in the clinical cases of elastofibromas, several central cords could also be covered by a common sheath (Fig 4) Alteration in the elastic tissue was mainly located in the fibrous bands, though not always, since similar changes could easily be observed in solitary elastic fibres running through fat (Figs. 6–8) as well as in the elastic elements of the walls of vessels passing through the fat.

In addition to augmentation of collagenous fibres and changes in the elastic fibres, several other alterations in the tissue deserve comment (Table 4). Necrotic foci were frequent and were not limited to areas with changes of elastic elements characteristic of clinical elastofibroma, but were found at least equally often in tissues without elastic degeneration. Necrosis was encountered in fibrous connective tissue, fatty tissue, and muscles. The necrotic areas measured up to 2 cm in diameter.

Necrosis of fibrous connective tissue was associated with an increased intensity of staining and swelling, rupture and disintegration of the fibre bundles. The nuclei were not stainable (Figs. 9, 10). When necrosis occurred in thus far unaltered areas, broken stumps of elastic fibres of normal thickness could be seen in the debris (Figs. 9, 10, 25). In the case of fresh, total necrosis of an artery homogeneous necrotic areas staining grey by Verhoeff's method were seen in the vicinity. Within fibrous bands, small wavy foci might occasionally be found which, using Verhoeff's stain often were of a smeared and dirty appearance. These formations were also encountered within clinical elastofibromas. We are of the opinion that these alterations represent a collagenous degeneration resulting probably from a break of the bundle (Fig. 11).

Necrosis occurred at least as often in fat as in collagen even if fresh necrotic foci were rare. In fresh necrosis, homogeneous, pale staining material similar to that found in necrosis of collagen, was seen in fat lobules (Figs. 12, 22). The necrotic fat could be resorbed by macrophages. Also a reduction of cellular fat content to multiple droplets, concurrent with decrease in the cell size, could occur (Figs. 13, 19, 23). In a more advanced stage, nothing but empty spaces, "fat cysts" could be demonstrated (Figs. 14, 15). These were often confluent, forming multilobular cavities (Fig. 14). Again the surrounding tissue could either be normal or show degeneration of elastic fibres. Sometimes the necrotic focus in fat was surrounded by a

Fig. 12. Fresh wall necrosis in the upper end of an otherwise nearly unchanged middle sized artery. Homogeneous necrotic foci also in neighbouring fatty tissue. No inflammatory reaction. Male, 90 years, with elastic degeneration (not seen in the picture). (Verhoeff's stain. × 110)

Fig. 13. Commencement of atrophy and necrosis of fat. In the centre of lobe, atrophic fat cells with several fat droplets. There is also infiltration by macrophages. Male, 61 years, without elastic degeneration. (Verhoeff's stain. × 106)

Fig. 14. Necrotic cysts with some debris in fatty tissue. Increased number of fibrocytes around some cysts, but no alteration of the elastic fibres. In the wall of the cyst, upper right, however, an augmentation of elastic fibres is likely. In the vicinity of the area illustrated, elastic degeneration was also demonstrated. Female, 61 years. (Verhoeff's stain. × 42)

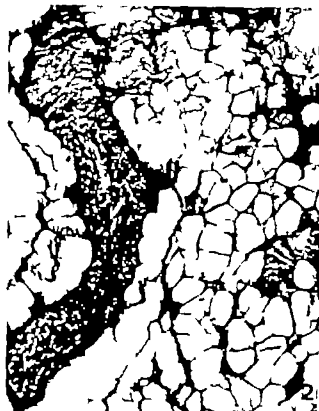
Fig. 15. Fat cyst framed by circle of swollen elastic fibres. In larger magnification, central cords and sheaths are easily discernible in the fibres. Some proliferated fibrocytes on the left. Cf. fig. 14 where a similar cyst without elastic degeneration is illustrated. Female, 79 years. (Verhoeff's stain. × 175)

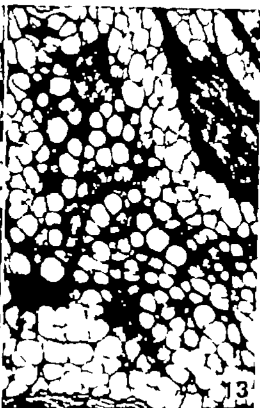
Fig. 16. Part of a fairly recent necrosis probably consisting mostly of muscle tissue and measuring about 1.5 mm in diameter. In upper part, two foci where the muscle fibres are still recognizable. Also in other parts of necrosis, clumps of debris are seen, probably derived from muscle. Some macrophages also occur. To the left, degeneration of elastic fibres, to the right, the tissue is normal. Female, 80 years. (Verhoeff' stain. × 42)

Fig. 17. Partial necrosis of the muscle fibres. Some of the fibres are swollen and disintegrating, in others cross-striation is preserved. There is pyknosis and caryorrhexis in the damaged areas. Male, 59 years, with elastic degeneration. (Verhoeff's stain. × 275)

Fig. 18. Fibrous scars in tendon tissue. Elastic fibres are lacking in collagenous scars, which show many fibrocytes. Female, 83 years, with elastic degeneration (no degeneration in the picture). (Verhoeff's stain. × 150)

Fig. 19. Cellular scar tissue replacing fatty tissue. The fat lobes and cells are decreased in size. Broken coiled elastic fibre fragments are embedded in the scar tissue. Uppermost, a small vein, lowermost capillaries. Female, 44 years, without elastic degeneration. (Verhoeff's stain. × 105)


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network of swollen elastic fibres in which the central cords, their coverings and also segmentation were clearly observable (Fig. 15)

While necrosis of collagenous and fatty tissue was common, necrosis of muscular tissue was more seldom seen perhaps because muscular tissue was not systematically examined. The necrotic areas contained other tissues as well as muscular tissue (Fig 16) and could measure about 2 cm in diameter All different stages of necrosis were observed In fresh necrosis the muscle fibres were swollen, their cross-striation had disappeared and their cytoplasm was variably clumped and featureless or showed more advanced disintegration (Fig 17) In other cases a gradual atrophy *of the musculature may* occur The fibres could become narrow and, occasionally sarcoplasmatic giant cells with several nuclei accurred as a proof of atrophy Often the muscle border was also irregular and muscle fibres, either isolated or in small bunches, were often present and intermingled with the fatty tissue.

Sometimes changes in nerves were also noted, such as disintegration of the myelin sheaths and even axons, and increase of fibrous peri- and endoneural tissue.

Augmentation of collagenous scar tissue is certainly one of the results of necrosis and ruptures of collagenous fibres (Figs. 18 19) In such areas, fibrocytes proliferate and produce a tight networks of unorganized fine collagenous bundles. Some convolutes of broken elastic fibres are often enclosed in the scar (Fig 19) Sometimes, however new formation of elastic fibres also seems to occur (Fig 20)

Cleft formation with tissue destruction may however be a much more common sequel to more extensive necrosis whether of collagen or fat than scar formation (Fig 21) Clefts, often of a length of several centimeters, may join to form still larger cavities. Necrotic tissue debris is often found on the margin or in the lumen clefts (Fig 22) When covered by a low mesothelial cellular layer *the clefts were transformed into bursae* (Figs. 24 25) It might be relevant to point out that cleft or bursa formation does not necessarily imply any elastic degeneration of the tissue.

It is characteristic of all these different kinds of *necroses* that inflammatory reaction was lacking or minimal. At times, however very oedematous areas could be found (Fig. 23) Lymphocytic infiltration, mainly perivascular was a rare occurrence, and only seldom were there aggregations of lymphocytes, histiocytes and polymorphonuclear cells in direct association with a necrotic area.

Particular attention was paid to the condition of the vessels. In arteries there were breaks in the elastic layers, *most often* in the elastica interna, intimal fibrosis and atrophy and scar tissue *in the media (Figs* 26 27) Sometimes calcification was also noticed in the media of the bigger arteries Where injury was widespread, vasa vasorum could be found to invade the damaged wall and also lymphocytes and histiocytes might occasionally infiltrate the arterial wall. In one person, a fresh almost complete necrosis of

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Fig 20 Seelns of very thin, probably newformed, elastic fibres in connective scar tissue. There is, however no very close contact between fibrocytes and the fibres. *The straight thick fibres are remaining* old elastic fibres. Female, 56 years, without elastic degeneration. (Verhoeff's stain. × *260)*

Fig 21 Four parallel clefts with necrotic margins *of ruptured* collagen bundles. No elastic degeneration except in the lower corner where massive globular degeneration has occurred. In the septa, fibrocytes have proliferated. Small arteries and veins unchanged. Female, 77 years. (Verhoeff's stain. × 42)

Fig 22 Early bursa formation also affecting necrotic fatty tissue. The wall of bursa is covered with a layer of amorphic substance. The elastic fibres in the vicinity of the bursa are not changed. Male, 59 years, with elastic degeneration. *(Verhoeff's stain. × 105)*

Fig 23 Oedema in connective and fatty tissue. The originally solid collagenous streak crossing the picture obliquely is totally dispersed. The fat lobes are atrophic and contain cells with multiple droplets. Male, 79 years, without elastic degeneration. (Verhoeff's stain. × 105)


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Fig. 26 A large artery showing discontinuity of the elastica interna (especially to the *left*) Some atrophy and fibrotic *foci in the* media and also invasion of vasa vasorum (lower part of fig.) No elastic degeneration in the artery Female 77 years, with elastic degeneration. (Verhoeff's stain. × 115)

Fig. 27 An injured *artery the* elastica interna and media of which is only partially preserved. Inflammatory cells and some vasa vasorum invade the wall, *but no* signs of elastic degeneration. To the left, a moderately large vessel with endothelial cover containing debris, presumably *lymphatic* (only partly seen in the picture) To the right, early scar formation with increased number of fibrocytes. The elastic fibres are normal. Same case as fig. 26. (Verhoeff's stain. × 105)

Fig. 24 Connective *and fatty* tissue necrosis and early bursa formation with mesothelial *lining*. Extensive globular degeneration of the elastic fibres in the vicinity Some fragments of unchanged elastic fibres in necrotic tissue. Female 83 years (Verhoeff stain. × 60)

Fig. 25 Part of a bursa formation. The wall is covered with mesothelium. Many nuclei in the collagenous *layer of the wall*. In the lumen, necrotic tissue fragments with stumps of unchanged elastic fibres. In the lower part of picture, the elastic fibres in fatty tissue are degenerated. Female 83 years. (Verhoeff's stain. × 60)

several arterioles was noted (Figs. 12, 28, 29) The smooth muscle fibres in the media were very swollen and homogenous and stained *light* grey by Verhoeff's method. Often *necrosis* extended into the intima as well. Sometimes the *endothelium* was spared and blood corpuscules could still be found in the lumen (Fig. 29)

The elastic elements of arteries very seldom showed any signs of hypertrophy and segmental degeneration, though adventitial fibres





Fig 26 A large artery showing discontinuity of the elastica interna (especially to the left) Some atrophy and fibrotic foci in the media and also invasion of vasa vasorum (lower part of fig.) No elastic degeneration in the artery Female, 77 years, with elastic degeneration. (Verhoeff's stain. × 113)

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Fig 24 Connective and fatty tissue necrosis and early bursa formation with mesothelial lining Extensive globular degeneration of the elastic fibres in the vicinity Some fragments of unchanged elastic fibres in necrotic tissue. Female, 83 years. (Verhoeff's stain. × 60)

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Fig 28 Fresh homogenous muscular necrosis of the artery wall. The wall is heavily swollen, packed erythrocytes and leucocytes are still perceivable in the lumen. No elastic degeneration is seen in the picture. Male 90 years, with elastic degeneration. (Verhoeff's stain. × 75)

Fig 29 Necrotic artery in muscle tissue. In the middle to the right, a small lumen with preserved endothelium, some intimal structure and some nuclei in the media. The plasma of medial muscle cells is [illegible] and homogenous. Sparse, very thin unchanged elastic fibres cross the wall. At the bottom a ne[illegible]ic arteriol. Elastic fibres in the vicinity are normal. Same case as fig 28. (Verhoeff's stain. × 450)

Fig 30 Cross section of two veins with marked fibrosis of the wall. No muscle cells are left. In the larger vessel probably some increase of the adventitial elastic network, in the smaller one (lower right corner) elastic network is scanty also in adventitia. In the lumen, packed partly destroyed erythrocytes and leucocytes which are better preserved. Male, 62 years, without elastic degeneration. (Verhoeff' stain. × 260)

Fig 31 Wall fibrosis of a small vein. Most of the wall is necrotic, uppermost endothelium still preserved. No proliferation in the adventitial elastic fibres. Male 66 years, without elastic degeneration. (Verhoeff's stain. × 260)

Fig 32 Branching veins with heavy proliferation of the adventitial elastic coat (in the middle). The lumina are narrow. To the left, an artery of normal structure, in the upper right corner a smaller artery and, further down, muscle tissue. Female, 80 years, without elastic degeneration. (Verhoeff's stain. × 105)

Fig 33 A vein with very marked fibrosis of the wall. No muscle cells occur. The elastic fibres are unchanged in the centre, but severely affected at the periphery of the vessel displaying a central cord and hypertrophic cover and also showing globular fragmentation. In the vicinity of the vessel, elastic fibres are unchanged. Female 75 years. (Verhoeff's stain. × 270)


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Fig 34 Capillary-sized vessel surrounded with elastic ring Amidst the hypertrophic elastic coat the original fibre can be discerned as a darker staining extremely thin cord. Female 79 years (Verhoeff's stain. × 450)

Fig 35 Tagential section of a small vein with fibrosis and hypertrophic adventitial elastic coat. In the swollen elastic fibres, the original fibre can be seen as a central cord. In the upper corner globular disintegration of the hypertrophic elastic fibres. Same case as fig 34 (Verhoeff's stain. × 270)

exceptionally might do This is in accordance with the experience from clinical elastofibromas where also the elastic cage of arteries, though not of veins, usually was saved (*Järvi et al.* 1969)

In veins two main types of changes might be seen which usually but not always, occur together in the larger vessels. Often a profuse intimal and medial fibrous proliferation was found to be the only change in the veins, which greatly narrowed the lumen (Figs. 10 30) Muscle fibres ld usually not be detected. More rarely els were found where a network of swolle lastic fibres in the adventitia with centr d and hypertrophic sheath was the onl thological finding (Figs. 34 35) In the larger vessels, both intimal fibrous and hypertrophic degeneration of the elastic fibres was a very common finding (Fig 33) already described in the report on clinical elastofibromas (*Järvi et al.* 1969) Fresh necrosis was also seen In venules (Fig 31) and the lumen was sometimes obstructed due to the swelling of the walls. True thrombi could not be found in the vessels, but often the lumen was blocked by blood cells, in particular by erythrocytes which seemed to be fused together thus forming a grained or amorphous material (Figs 28, 30) This picture may indicate a permanent stagnation of the circulation In the vessel.

Owing to the enormous hypertrophy of the

fibres in the adventitia it is very difficult to estimate whether new formation of the elastic fibres also plays any part in the changes in veins. However within the massive fibrotic proliferation of the walls there were often quite a number of thin elastic fibres among the collagenous fibres (Fig 33) the original presence of which hardly is conceivable. Occasionally vessels may be found in which the adventitial elastic network is markedly increased though without any sign of hypertrophy or degeneration (Fig 32) Such findings support the concept of the capacity of connective tissue to produce new elastic elements under certain conditions, even in adult life.

In the course of examination of the slides, the impression may be obtained that a more general increase of both fibrocytes and collagenous fibres occurs along with all the necrotic changes described above. The fibrous bands are thicker than usual and, between the *apparently older organized collagenous* bundles, thinner bands without any distinct architectural order may be found. This unorganized fibrous connective tissue, rich in fibrocytes, invades the connective layers of fasciae, replaces the fatty tissue between the latter *and encloses the muscle tendons. An increasing* number of capillaries in fat lobes may join the process, perhaps leaving small fat islands inside the proliferating collagen as remnants (Fig 19) The augmentation of collagenous tissue seems to be one of the earliest changes since it often can be detected alongside intact elastic fibres. This newly formed tissue differs from scar tissue only insofar as the original connective tissue in scars is destroyed.

TABLE 4 *Occurrence of Different Alterations in the Subscapular Tissue and Thoracic Fascia in Cases of Subclinical Elastofibroma and Cases without Elastic Degeneration. In Brackets Number of Additional Cases with Very Mild or Uncertain Changes. These Cases Have Not Been Counted in Percentages*

	Elastic degeneration 39 cases		Without elastic degeneration 196 cases	
	No. of cases	per cent	No. of cases	per cent
Connective tissue Fibrosis	31 (3)	80	80 (20)	41
Necrosis with clefts	29	74	52 (16)	27
Fat Cysts	32 (1)	82	112 (37)	57
Fresh necrosis	2	5	1 (4)	0.5
Atrophic and disappearing fat lobes	13	34	45	3
Muscle Atrophy	5 (2)	13	10 (43)	5
Necrosis	12 (1)	31	47 (12)	24
All tissue necroses together	29 (2)	74	74 (16)	38
Bursae	18	46	9 (5)	5
Bursae and necrosis together	33	85	76 (17)	39
Arteries Small lesions	17 (2)	44	28 (3)	14
Necroses of the wall	1	3	1	0.5
Elastic degeneration	3	8	—	—
All damages together	17 (2)	44	28 (3)	14
Veins Wall fibrosis	31 (3)	80	70 (27)	36
Necrosis of the wall	9	23	24 (7)	12
Wall fibrosis and necrosis together	31 (3)	80	73 (26)	37
Elastic degeneration	28 (3)	72	—	—
All venous lesions together	35 (2)	90	73 (26)	37
Arteries and veins together Vascular lesions except vascular elastic degeneration	32 (2)	82	75 (28)	38
Vascular elastic degeneration included	35 (2)	90	—	—

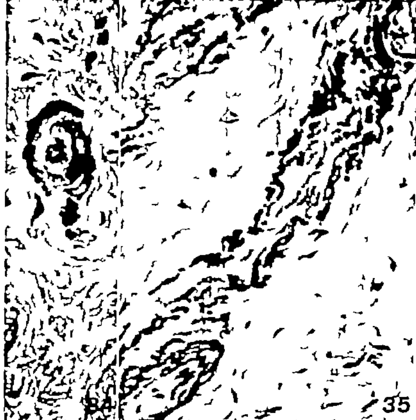


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All tissue necrosis together	29 (2)	*74*	74 (16)	*38*
Bursae	18	*46*	9 (5)	*5*
Bursae and necrosis together	33	*85*	76 (17)	*39*
Arteries Small lesions	17 (2)	*44*	28 (3)	*14*
Necrosis of the wall	1	*3*	1	*0.5*
Elastic degeneration	3	*8*	–	–
All damages together	17 (2)	*44*	28 (3)	*14*
Veins Wall fibrosis	31 (3)	*80*	70 (27)	*36*
Necrosis of the wall	9	*25*	24 (7)	*12*
Wall fibrosis and necrosis together	31 (3)	*80*	73 (26)	*37*
Elastic degeneration	28 (3)	*72*	–	–
All venous lesions together	35 (2)	*90*	73 (26)	*37*
Arteries and veins together V scular lesions except vascular elastic degeneration	32 (2)	*82*	75 (28)	*38*
Vascular elastic degeneration included	35 (2)	*90*	–	–

Histological Alterations in Cases without Elastic Tissue Degeneration

All tissue changes which occurred in association with elastic fibre hypertrophy and degeneration were also found in the absence of the latter (Table 4) Most frequent were the fatty cysts, empty round spaces in paraffin embedded sections and larger than the fat cells, occurring in fatty tissue but sometimes also in collagenous tissue. Sometimes these cavities might be filled with a fluid other than fat. Some isolated cavities may also represent technical artefacts, but a more frequent occurrence certainly suggested a pathological change. The broadening of fascial collagenous layers with newly formed scar like, unorientated collagenous tissue was also a very common alteration leading to corresponding decrease and disappearance of fatty tissue.

Changes in the vessels were observed even in the early stage occasional breaks and defects in the elastic network, particularly in the internal elastic lamina in large arteries, combined with small foci of intimal and medial fibrosis and larger and more continous fibrosis of the media and intima of the middle-sized and small veins often leading to complete necrosis of the vessel. Vascular lesions were found in 38 per cent of the cases without elastic tissue changes, and in 90 per cent of the cases of subclinical elastofibroma. Oedema, collagenous, fatty and muscular necrosis, degenerative changes in nerves, cleft and bursa formations were found in association with increased collagen and the vascular changes, but as in cases of subclinical elastofibroma, these findings were more irregular Necrotic clefts were seen in 27 per cent of the cases without elastic tissue changes, muscular damages in 24 per cent, bursae in 5 per cent, corresponding to 74 per cent 31 per cent and 46 per cent in cases of subclinical elastofibroma. The vascular and necrotic changes were mostly but not always, concurrent, as apparent from the figures applying to the cases in question in table 4 Among cases not showing el stic changes, necrotic and/or vascular lesion were manifest in 53 per cent all except one of the cases presenting elastic hypertrophic-degenerative change showed one of these lesions or both. It is probable that a more careful scrutiny of the cases could have brought the concordance still closer

If the different kinds of necrosis and bursae are considered indications of a more severe damage of subscapular tissue, signs of severe lesion were to be found in 39 per cent of cases not showing changes in the elastic tissue and in 85 per cent of those presenting subclinical elastofibromas (Table 1) Two females under 50 years belonged in the former category One was a 30-year-old saleswoman, the other a shopkeeper of 44 years. Both showed fibrosis and also necrosis in veins with scarring of connective tissue and corresponding reduction of fat lobes. In the latter case, atrophy of nerves and muscles also occurred. The sex distribution of lesions gives a rather controversial result since the severe lesions occur more often in men (in 39 per cent) than in women (27 per cent) whereas clinical as well as subclinical elastofibromas clearly were more frequent among women. As mentioned no reasonable explanation of this finding can be given.

DISCUSSION

The different kinds of changes found in the cases of subclinical elastofibroma necrosis of collagenous- fatty- and muscular tissues, and arterial walls obstruction of the vessels with fibrosis widespread hypertrophy and degeneration of the elastic elements degeneration and fibrosis of nerves formation of scars, clefts, cavities and bursae suggests that the primary pathology of the disease may be damage to blood vessels. The rupture of elastic elements and necrosis in the walls, atrophy and scars in the media of arteries and obstructive fibrosis of veins may be the result of streching and mechanical injury caused by friction of the scapula and must be accompanied by local nutritional deficiency leading to oedema and acute tissue necrosis.

Such necroses can affect normal tissue as well as already damaged tissue. Depending on the location and extent of necrosis, a scar cyst or bursa may be the final result of the nutritional deficiency. Hypertrophy and degeneration of elastic fibres could be explained as the consequence of circulatory insufficiency of minor degree. If so, the elastic elements should be considered relatively sensitive to nutritional failure though possessing a marked capacity for regeneration.

Further consideration of the histological picture as a whole and the quantitative relationships of the different changes raises doubts about a sequence of pathological alterations as simple as that suggested above. Quantitatively proliferation of fibrocytes and collagen as well as the elastic hypertrophy and degeneration are the predominant alterations in most cases, and the importance of changes in vessels seem to lag behind these processes. For example, fresh total necrosis of an artery was noted only in two cases in this series. In the remaining cases there was not even evidence of a past history of necrosis. On the other hand, collagenous proliferation as well as hypertrophy and secondary degeneration of elastic tissue was often the only visible change in the tissue and hence, they could not be directly dependent upon each other. Accordingly it seems probable that fibrocyte proliferation with new collagenous fibre formation, hypertrophic-degenerative alteration of elastic tissue and damages to the vascular system are all largely independent, though having the same common cause of overstrain of the tissue due to friction of the scapula during movements of the upper extremities. Thus, the increase of fibrocytes and collagenous fibres would constitute a response of fibrous connective tissue and hypertrophy of elastic fibres of elastic tissue. Both these processes may also occur in the walls of veins, whereas more acute damage such as rupture of the elastic network and necrosis seems to be the rule in arteries. The development of acute necrosis, formation of clefts, cysts and bursae as well as disintegration of the hypertrophic elastic fibres in globes and debris may however still be of secondary nature and based on circulatory and nutritional deficiency. The production of new elastic fibres is also a possible component of the connective tissue response mentioned above, but is difficult to estimate. Evidence of an increase in elastic fibres in some veins which showed fibrotic proliferation of their walls was more convincing.

The widespread occurrence of elastofibroma like changes in older people indicates clearly the non-neoplastic character of the disease, as do the many regressive alterations associated with it. This also suggests that the development of the lesion into clinically demonstrative elastofibromas must be slow and take a relatively long time. The predominant location—although not very marked—of the lesion on the right side agrees with the assumption of an occupational aetiology of the disease. The clear preponderance of females among the affected people is to some degree surprising but perhaps more women than men are employed in repetitive manual work. However the other degenerative changes in contrast to elastic alterations were more frequent in men than in women, thus contradicting this hypothesis. On the other hand, the frequency of bilateral lesions, the occurrence of the lesion in the absence of special occupational stress is to be demonstrated, and the appearance of some clinical tumours in early middle age indicate the role of constitutional factors in the pathogenesis. In this connection, small variations in the shape of the scapula could be of some importance. To evaluate this condition, dissection of the scapula would be necessary but was not done in this study.

It is very unlikely that the extensive tissue damage found in cases of subclinical elastofibromas, such as areas of necrosis 2 cm in diameter failed to cause any pain or aching, even though patients with clinical elastofibromas seldom complained of pain. One of our patients seen most recently however had visited the doctor because of acute pain in the back. Massage is a popular treatment of such pain in the back in Finland, and might

well aggravate the lesion rather than alleviate and cure it.

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FIBRINOID NECROSIS OF LIVER PARENCHYMAL CELLS

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By means of different histological and histochemical investigations and fluorescence microscopy it has been possible to demonstrate fibrinoid necrosis of parenchymal liver cells in cases of delayed chloroform death and in septic, toxic and infectious hepatitis. This type of necrosis has previously been observed only in connective tissue and in vessel walls, in particular in collagenoses. It was also possible to provoke this type of liver cell necrosis in animal experiments. Reference has been made to differences in the chemical composition of the fibrinoid substance and to relations to other related substances (amyloid, keratin, fibrin). The importance of fluorescence microscopic investigations with fluorochromes, using *Nevelli's* method and with certain other procedures is emphasized.

Key words: Fibrinoid necrosis; liver cells.

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Since the appearance of the works of *Wuhrmann & Wunderly* (38), *Klemperer* (16) and others (1, 39, 17) who showed the importance of the paraproteins, the changes of the ground substance of the connective tissue, and the role played by different "protein debris" in certain types of diseases, the attention paid to these substances has increased steadily. The research has concentrated mainly on amyloid, fibrin, fibrinoid and keratin substances.

It applies in general that it is only the fibrinoid substances in connective tissue and in vessel walls which have been described and been shown to be important constituents in the pathological processes in collagenoses. Our present histological and histochemical investigations indicated, however, the possibility that fibrinoid degeneration and fibrinoid necrosis may occur also in the parenchymal cells of the liver.

As the possibility of producing experimental fibrinoid necrosis implied a great progress in research (*Movat* 23, *Jellinek* 15) we also tried to demonstrate this peculiar type of necrosis of the liver cells in animal experiments.

METHODS

Fixation in formaldehyde. Routine staining with haematoxylin-eosin and van Gieson's stain. Staining according to *Ivemark's* modification of Ludewig's method (14). Taft's staining as modified by *Dahl's* staining 1 h at room temperature, 1 h at 35 C, and at room temperature overnight (8). Staining after Goldner, Weigert's and Kockel's fibrin staining (18). Heidenhain iron-alum-haematoxylin (13), the PAS reaction, alcian blue, Congo red after Bennhold, methylgreen-pyronine, PTAH, and orcein staining. Acridine orange and Thioflavine S were used as fluorochromes for fluorescence microscopy. The sections stained with haematoxylin-eosin and with all the other methods were also

investigated in Wood light (24). For an excellent criticism of the staining methods for amyloid and fibrin, see *Brandt* (5).

SERIES OF PATIENTS AND RESULTS

In order to facilitate the description of the results, these are summarized in tables where only the stains giving either positive or important negative results are included. Van Gieson's stain is omitted since it always only caused a faintly red fluorescent yellow stain or no staining at all. Similarly results of staining with alcian blue are omitted in the tables since this method never revealed the presence of acid mucopolysaccharides (MPS). The same applies to PTAH and mucicarmine stainings which often give unreliable results. The crosses in the upper line indicate that staining is observed in the light microscope, while the lower line in each square shows the intensity of the fluorescence in the same slide. The empty spaces imply that the investigation has not been carried out—due to insufficient supply of material.

Case report 1. A 13 years old boy was taken ill with influenza; a wide-spread bronchopneumonia in the right side and violent pains in the right hypochondrium were manifest. The appendix had to be removed on the fifth day of the illness. Chloroform anaesthesia was given. After the operation the patient recuperated and there was no fever. Three days later the fever recurred, the patient felt sleepy and later restless. On the fourth day fits and slight icterus occured, and on the fifth day coma and death ensued. Post mortem examination revealed acute tracheobronchitis and swelling of the mediastinal lymphglands. The liver was slightly enlarged; the cut surface displayed a distinctive pattern, the centre of the lobuli being reddish brown, the edges yellow.

Case report 2. This 10 years old boy was taken ill with a disease reported as mushroom poisoning and with serious gastro-enteritis. Two weeks later tonsillitis and violent earache suddenly occurred. Because of high fever trepanation of both processi mastoidei was carried out under chloroform anaesthesia. No fever was noted for three days. On the fourth day there was high septic fever, later fits, and slight jaundice. Death in coma ensued five days after the operation. Post mortem examination showed septic changes and slightly enlarged liver with similar but less pronounced changes as in the first case.

The histological investigation of liver sections of *case no. 1 stained with haematoxylin-eosin* revealed extensive necrosis of the parenchymal cells in centrolobular regions. Eosin gave an intensive red staining to the necrotic cells. In the centre of the lobuli stasis was observed and a local increase in the number of Kupffer cells. At the boundary between the necrosis and the normal liver tissue in the periphery of the lobuli there was a zone of cells presenting minute vacuoles in their cytoplasm ("ballooning cells") (28, 40).

This change was termed "cell hydropsy" by *Fischer Wasels* (9) and "urticaria of the hepatic cells" by *Pribram & Upham* (27). It has been observed in "shock liver" by *Endes* (12). The cause of the change is according to the majority of the authors the changed circulation of the diseased liver. The observations by *Anrorth et al.* (2) based upon electron microscopy indicate that most of the vesicles may originate from vacuolar expansion of the endoplasmic reticulum. The injured cells appear to loose their ability to eliminate water.

The necrotic cells were partly disengaged and were located freely in the sinusoids. The strongly eosin-stained cells exhibited a pronounced golden-yellow fluorescence against a rather dark environment. The vacuolated cells did not fluoresce. Congo-red displayed a red colour of the necrotic cells, quite similar to ordinary amyloid. The congo-positive cells showed a red fluorescence, but were not birefringent as amyloid. Using azan staining and Masson's and Ladewig's trichrome staining, the damaged cells became intensely red to brownish red and lacked fluorescence (Fig. 1). The PAS reaction imposed a distinct red colour on the necrotic liver cells with slight reddish fluorescence. The affected liver cells were not stained by methyl-green-pyronine but displayed a very pronounced silver-white fluorescence with a pale rose-coloured tint. Thionine and toluidine blue did not stain the damaged cells but a sky-blue luminescence (i.e. strengthening of the autofluorescence) was quite pronounced. The sections stained according to Goldner's method displayed a reddish brown discolouration of the altered cells which clearly distinguished these from the environment; no fluorescence was noted. Using Taft's method, the necrotic cells were somewhat deeper red than the other ones. They displayed, however, a strikingly golden-yellow fluorescence in Wood light (Fig. 2).



Fig. 1. Case 1. Delayed chloroform death with centrolobular liver cell necrosis. Vacuolated cells in the surroundings. *Ladewig* stain. × 125.



Fig. 2. Liver from the same case, stained according to Taft. Fluorescent light. × 250.

This fluorescence was so strong that so-called single cell necroses were distinctly visible in the adjoining areas of normal appearance. This method has turned out to be the best by which to find the necrotic cells. Weigert's stain was negative, an autofluorescence was, however, visible in the damaged cells. The fibrin staining after Kochel, and Heidenhain's iron-haematoxylin as well as the PTAH staining were strongly positive in these cells. Mucicarmine was faintly positive, giving a faint glow in Wood light. Staining with acridine orange displayed a bright yellow fluorescence in the affected areas as compared to the weak reddish-yellow fluorescence of the environment. Thioflavine S caused a silver-blue fluorescence of the cells in the central, altered areas, while the surroundings were dark.

Case no. 2 showed in general the same changes in the light microscope and by fluorescence methods as in case no. 1. One difference was noticed, however: the localisation of the necroses in the first case was centrolobular, but in the second case the necroses occurred in the intermediary zone. Thus, the deciding aetiological factor was probably the fungus poisoning, and the ensuing chloroform anaesthesia aggravated the severe septic condition (Table 1).

Because septical complications occurred in cases 1 and 2, two further cases with serious sepsis will be described.

Case report 3. Female, 63 years old, with collum fracture, gas phlegmone and sepsis. Died in pulmonary embolism. The liver was enlarged (2150 g) with diffuse change of the cut surface as in Case no. 1.

Case no. 4. Male, 23 years old, in septic condition with swelling of the liver and slight jaundice. The autopsy revealed purulent mastoiditis, the liver was enlarged (2300 g), the appearance of the cut surface being the same as in Case no. 3.

The changes in the liver were in principle the same as in the first two cases. Eosinophilic necroses of the liver cells in centrolobular areas and single cell necroses were observed. Circulatory disturbances and vacuolic cell degeneration were also common. It is remarkable that staining with acridine orange did not cause any fluorescence, while thioflavine S yielded a silver-white fluorescence (Table 1).

The following cases serve as examples of liver cell necroses after serious circulatory disturbances and hypoxia as encountered in the presence of thrombi in the large liver vessels.

Case report 5. Male, 67 years old, with periarteritis nodosa, thrombi in the branches of the portal vein and the hepatic artery. Necroses, similar to infarcts, in the liver were observed at autopsy.

Case report 6. Male, 22 years old, with circulatory insufficiency and thrombi in the femoral vein, vena cava inferior, the hepatic veins and the portal vein. Necroses in the liver, similar to infarcts, were found at autopsy.

TABLE 1 *Fibri*

	Haem. eosin	Ladewig	Goldner	PAS	Congo red
Delayed chloroform death	+ + +	+ + +	+ + +	+ +	+ + +
	+ + +	—	—	+ + +	+ +
Sepsis	+ + +	+ + +	+	+ +	+
	+ + +	—	—	+	+ +
Thrombosis of the v portae	+ + +	+ + +	+ +	+ +	±
	+ + +	—	—	+	±
Toxic hepatitis	+ +	±		+ + +	—
	+ + +	—		+ + +	—
Inf hepatitis with fatal outcome	+	±	—	±	—
	+ + +	—	—	+ +	—
Inf hepatitis diag puncion	+	±	+ +	—	—
	+	—	—	±	—
Exp. chloroform poisoning 1–3 days	+ + +	+ + +	+ + +	+ +	±
	+ + +	—	—	+ +	±
Exp. chloroform poisoning 4–6 days	+ + +	+ + +	+ +	±	+ + +
	+ + +	—	—	+ +	+
Experiments in magnetic fields	+ +	+ +	+ + +	+	—
	+	—	—	—	—

Case report 7 Female, 77 years old, with bile duct cancer and thrombi in the branches of the portal and hepatic eins. Necroses in the liver similar to infarcts were observed at autopsy

In these cases, centrolobular eosinophilic necrosis with strong fluorescence were noted. The secondary fluorescence with acridine-orange was not stronger than that of normal liver cells; however a different colour occured. Thus, in the latter a golden-yellow fluorescence was obtained, while the necrotic cells had a yellowish-green colour (25 36) Thioflavine S staining resulted in a silver-blue fluorescence against a sky-blue background (Table 1)

In the last group we investigated one case .. toxic hepatitis and some cases of infectious hepatitis.

Case report 8 Female, 54 years old, with urinary tract infection treated w th furadantin. During the treatment, pathological liver tests were observed and a liver biopsy was carried out. The clinical diagnosis was "toxic hepatitis?" Sections stained with haematoxylin-eosin showed dilated eins with stasis. No centrolobular necrosis was visible, but minute groups consisting of 10–20 liver cells with poorly stained nuclei and a pronounced eosinophilic cytoplasm were observed In Wood light, a strong golden-yellow fluorescence of the necrotic foci was observed. Thus, the diagnosis of toxic hepatitis could be corroborated (35)

Further cases refer partly to patients dying of infectious hepatitis with symptoms of liver insufficiency and to patients biopsied for diagnostical purposes during the progress of the disease.

Case report 9–10 Autopsy material from patients dying of li er coma and generally displaying complete necrosis in large areas of the liver and signs of regeneration in others The histological signs of fibrinoid necroses in the liver cells were not very striking; however eosinophilic single cell necroses with rather strong fluorescence were sometimes encountered. Sections stained according to *Ladewig's* method or treated according to Goldner showed positive staining of a larger number of liver cells than otherwise seen. There might be seen a multicoloured aspect of reddish-lilac-blue cells in a previous paper (31) called a "mosaic structure" and by *Mladyk* (22) termed "piebald" pictures Using Kockel's and Heidenhain's method the Councilman bodies were darkly coloured.

Many biopsied cases from the epidemic in *Stockholm* in 1964 belong to this group. Histologically they displayed typical pictures of hepatitis with eosinophil necrosis of some liver cells, and scattered small groups of vacuolated cells. The eosinophil necroses showed a golden-yellow fluorescence. Ladewig's staining showed reddish colour or reddish deposits in the necrobiotic cells. Goldner staining resulted in a bright red colour Fibrin

Methyl-green-pyronine	Taft	Weigert	Kockel	Heidenhain	Thionine	Acridine orange	Thioflavine S
—	+ + +	—	+ + +	+ + +	±	+ + +	+ + +
+ + +	+ + +	+ +	—	—	+ + +		
—	+	—	+ +	+	+	—	+ +
+ +	+ + +	—	—	—	+ +		
—	+ +	—	+ + +	+	+	+	+ +
±	+ + +	—	—	—	+		
—		—	+	±	+	+ + +	+ + +
±		+ +	—	—	—		
—	—	—	—	±	—	+	+
+	—	±	—	—	+ +		
	+	—	+	+	—	+ + +	+ +
	±	—	—	+	—		
±	+ +	—	+ + +	+ +	±	+ + +	+ + +
+	+ + +	±	—	—	+ +		
±	—	—	+ + +	+ + +	—	+ + +	+ + +
±	+ +	—	—	—	+ + +		
+		—	+ +	+ +	—	+ + +	±
+		±	—	—	—		

stains and fluorochromes revealed positive results in single cell necrosis (Table 1)

The results in this group are not so prominent as those previously described. The morphological phenomena depend, however to a great extent on the kind of virus in question and on the time of onset of the illness (25 40) The cells exhibiting total necrosis have already passed the state of fibrinoid necrosis

Animal Experiments and Results

In the subsequent investigations we have tried to produce experimentally the peculiar necrosis of the liver cells described above. Dogs, and particularly rabbits and mice are sensitive to chloroform. Only the experiments carried out on white mice will be discussed in this paper while the remaining ones will be mentioned briefly

1 In experiments carried out in collaboration with Ask Rochard & Ylinen (19 20) white mice were exposed for 4 hrs to tri- and tetrachlorethylene in fume chamber of about 15 l volume. As control, *chloroform* was used The mice were exposed to 100 200 400 and 800 ppm. and killed after 1 2, 3 4 and 5 days After one day the animals treated with 800 ppm. of chloroform showed necrosis of the liver some already at 200 ppm. Acidophilic necroses were observed around the central veins and were surrounded by vacuolated cells, exactly as in the human cases (*Fig* 3) Within 1–3 days the affected cells give faintly positive PAS reaction with a prominent rose coloured autofluorescence, while congo-red staining is positive only after 4–5 days. At that time the PAS reaction disappears almost completely Thioflavine S staining yields a very intense light blue fluorescence on a dark background (*Fig* 4) (See Table 1)

2. White mice were investigated in collaboration with *J & M Barnothy* (3 4) in order to study the influence of magnetic fields on the organs.

The mice were kept for 15 days in a vertical, homogeneous magnetic field of 9000 Örsted, the controls being located in a dummy" magnet. Alterations were found in the adrenal glands, the bone marrow and the spleen. In some cases it was possible to find small groups of liver cells presenting acidophilic cytoplasm and even showing single cell necrosis. By Goldner staining red coloured cells were observed in a number much larger than that to be seen after staining with eosin. The cells stained positive if the Kockel, PTAH and Heidenhain methods were used (*Fig* 5) Concerning the results of the other staining procedures, see Table 1



Fig. 5. Liver of a mouse 2 days after chloroform inhalation. Vacuolated cells. Haematoxylin-eosin. × 450.

3. Earlier experiments were carried out in collaboration with *Goreczky & Roth* (31) on rabbits. To create a reliable and always comparable injury to the liver the animals were treated with subcutaneous doses of chloroform.

In this way it was possible to approach the pathogenesis of fulminant acute hepatitis by treating the rabbits in advance with phosphorus or phenylhydrazine or with a hepatotoxic immune serum. After complete recovery these animals were subjected to subcutaneous sublethal doses of chloroform. The results showed that the percentage of deaths among pretreated animals was much higher than that among animals in a control group and death occurred more rapidly in the former group. Since we were able to establish a preceding liver disease in 10 out of 13 cases of human fulminant hepatitis, we have presumed an auto-immune liver disintegration after a second blow. The histological changes showed the same centrolobular type of eosinophil necrosis as the ones described in the present paper after chloroform anaesthesia (cases 1 and 2).

In other experiments, a storage of copper of the diseased liver regions after chloroform treatment could be revealed, (30). *Mallory* believed this to be one of the aetiological factors of liver cirrhosis (21).

Thus it appears from all these investigations that a peculiar type of liver cell necrosis occurs which morphologically and histochemically corresponds to fibrinoid necrosis.

Since this previously has been observed only in the mesenchymal tissues and not in parenchymal cells, we have investigated certain pathological processes as controls in which a true "classical" fibrinoid degeneration or necrosis appears. Such processes are collagenoses, such a periarteritis nodosa and lupus erythematodes. The control cases also have to include changes associated with the secretion of "pure" fibrin. A detailed description has to be limited only to a couple of examples.

Fibrinoid Controls

Cases of periarteritis nodosa refer to biopsies from the skin, and the tonsils, and to autopsy cases (*Table 2*). By eosin and Taft's staining the diseased vessels are light red or faintly red, displaying a strong fluorescence. The PAS reaction is posi-

TABLE 2 *Control Cases* ...

	Haem. eosin	Ladewig	Goldner	PAS	Congo red
...riart. nc	+ +	+ + +	+ + +	+ +	+
...n	+	+ +	+ +	+	−
...riart. nodo-		+ + +			+ + +
...sal		+			+ +
...riart. nodosa	+ + +	+ + +	+ + +	+	+ +
...dney	+ + +	+ +	+ +	+	+ +
...riart. nodosa	+ + +	+ + +	+ + +	+	+ + +
...er	+ +	+	+	±	±



Fig 4 Liver of a mouse 4 days after chloroform inhalation. Thioflavine S stain—fluorescent light. × 250.

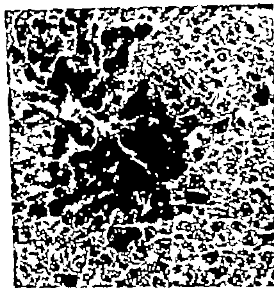


Fig 5 Central liver cell necrosis of a mouse kept for 15 days in a magnetic field. Heidenhain's stain. × 300.

tive in all cases, often quite strong, with a pronounced silver-red fluorescence. Ladewig's and Goldner's staining was positive in all cases (*Fig 6*). A distinct congophilic angiopathy was observed in four cases out of eight. The congophilic material was never birefringent, just a greyish red fluorescence was visible (29). Staining with thioflavine S resulted in fluorescence in the autopsy cases (*Fig 7*).

Fibrin Controls

These refer to cases of pericarditis, pleuritis and peritonitis fibrinosa, fibrinous pneumonias, tracheitis fibrinosa and skin ulcers with fibrinous coatings. Weigert's fibrin staining was always positive, which was never seen in the liver cases or in the fibrinoid necrosis of the collagen. Kockel's fibrin staining was positive, too, but Heidenhain's staining which always was positive in the liver cases could faintly be noticed only in one case of pneumonia (*Table 3*).

DISCUSSION

Delayed effects of chloroform have been described previously and discussed in detail and they have been observed in animal experiments by *Fischler* (10). The described changes in the liver are quite similar to those observed by us.

On the above basis we have formed the opinion that there is a kind of fibrinoid cell necrosis of the parenchymal cells in cases

ibrinoid Necrosis in the Vessel Walls

Methyl-green-pyronine	Taft	Weigert	Kockel	Heidenhain	Thionine	Acridine orange	Thioflavine S
—		—	+ + +	+ + +	—		
+		—	—	—	+ +	+ + +	+
—	+ + +	±	+ + +	+ + +	+		
+ +	+ + +	+ +	—	—	+ +	+ + +	+ + +
—	+ + +	—	+ + +	+ + +	+		
—	+ + +	+ +	—	—	+ +	+ + +	+ + +

	Haem. eosin	Ladewig	Goldner	PAS	Congo r
Fibrinous pericarditis	+ + +	+ + +	+ + +	+ + +	±
	+ + +	+	+	+ +	+
Fibrinous peritonitis	+ +	+ + +	+ +	+ +	—
	+ + +	+	±	±	+
Fibrinous pleuritis	+ +	+ +	+ +	+ +	—
	+ + +	—	—	+	—
Fibrinous tracheitis	+ + +	+ + +	+ + +	+ + +	—
	+ + +	+	—	+ + +	—
Fibrinous pneumonia	+ +	+ + +	+ + +	+ +	—
	+ + +	+ +	—	—	—
Ulcerations of the skin	+ + +	+ + +	+ + +	+ + +	—
	+ + +	—	—	—	—

of liver disease showing so called eosinophilic (acidophilic, coagulation) necrosis. The changes agree *fundamentally with those* which may be observed in fibrinoid necrosis of connective tissue in collagenoses.

Concerning the changes of the liver we would like to refer to some points.

a) As we have seen in the cases of experimental chloroform poisoning, a distinct PAS positive reaction in the liver cells appeared already after 1–3 days. By the same time the



Fig 7 Control case. Periarteritis nodosa. Fibrinoid necrosis in an artery of the liver. Thioflavine S stain in fluorescent light. × 1.5.



Fig 6 Control Periarteritis nodosa. Fibrinoid necrosis in an arte of the kidney *Goldner's stain.* × 125.

congo red staining was hardly visible. After 5–6 days the positive PAS reaction disappeared in spite of the fact that the fluorescence still remained visible. The congo-red staining on the other hand, quite definitely became positive. This behaviour is strikingly similar to that of experimentally produced amyloid, which in the beginning is also *PAS positive* and later could be stained only with *congo red* (17). Even the behaviour of the elastic amyloid is the same (32 33 34). Quite surprising is the coincidence of the two reactions

S cretion of Fibrin

thyl-green-pyronine	Taft	Weigert	Kockel	Heidenhain	Thionine	Acridine orange	Thioflavine S
±	+ + +	+ + +	+ + +	—	+ + +	+ + +	+ +
—	+ + +	—	—	—	—		
—	+ +	+ +	+ + +	—	+ +	+ + +	+ +
±	+ + +	—	—	—	—		
—	+ +	+ + +	+ + +	—	+ +	+ +	+
—	+ + +	—	—	—	—		
—	+ +	+ + +	+ + +	±	+ +	+ + +	+ +
+	+ + +	—	—	—	—		
—	+ +	+ +	+ + +	±	+	+ + +	+ +
±	+ + +	—	—	—	—		
—		+ + +	+ + +	±	+ + +	+ + +	+ +
+ +		—	—	—	—		

in cases of chloroform injury in man. These results and those of the other stainings indicate a change and increase of α-glycol groups and of the glyco- and neutral mucopolysaccharides (26). The control cases of mesenchymal fibrinoid showed similar results. The PAS reaction was only faintly positive in the affected vessel walls in cases of periarteritis nodosa, whereas congophilic material could be demonstrated in 50 per cent of the cases.

b) The results of the *fibrin staining methods* indicate the importance of the distribution of the fibrin and of the different fibrinoid substances. In the liver cases the results were always positive in the presence of chloroform injury sepsis and thrombosis. Often a brownish-red colour was noted while the fibrinoid necroses in the vessels showed a brilliant red colour by the same staining methods. This difference might be explained by the special content of bilirubin or lipofuscin in the liver cells, and possibly also by their content of pentosenucleoproteids (26).

c) Concerning the differences between fibrinoid and fibrin we would like first to mention the results of the staining with *thionine*. Fibrin was stained strongly by this dye, without any fluorescence at all. The necrotic liver cells displayed in general a very weak, hardly noticeable blue staining, but an intensified blue-silvery fluorescence. Vascular fibrinoid behaved in exactly the same way.

Another difference exists in the staining by *Heidenhain's haematoxylin*. By this method the staining of the liver cells became strongly positive after chloroform injury and in the experiments. The vessels in periarteritis nodosa were equally positive. The fibrinous exudate, on the other hand, was always negative or ± only.

In contrast, *Kockel's fibrin staining* was strongly positive in cases of chloroform injury sepsis and thrombosis, and was also positive in fibrinoid necroses of the vessels and in precipitated fibrin.

Of special interest were the results obtained by *Weigert's staining*, which, with the exception of fibrin deposits, always gave negative results. However, a rather strong fluorescence was observed in toxic hepatitis and in the vessels in cases of periarteritis nodosa. This could sometimes be observed also if Taft's— or staining methylgreen-pyronine-staining were used. This intensified autofluorescence might be termed a *dichroic fluorescence* and may be taken in support of the theory of a fibrin secretion and deposition at the very beginning of the process.

d) Regarding *fluorochromes*, fibrin and fibrinoid in the vessel walls and in the liver cells cannot be distinguished by acridine orange staining since both give a vivid golden-yellow fluorescence (blamed to indicate a disturbance of the RNA metabolism) (25, 36). The fluorescence of the slides stained with thioflavine S is in general paral-

lel to the acridine-orange staining It is quite strong for the liver cases and for fibrin, weaker however in fibrinoid necrosis of the vessels *except those with congophilic angiopathy* (see Fig 7)

These results indicate a difference in the chemical composition of the deposited material. Fibrinoid—like amyloid—is not a uniform, well-defined substance The presence and precipitation of mucopolysaccharides, originating from plasmaproteins must be considered also fibrin, fibrinogen and globulins may be present in varying amounts and distribution (23) The relationships between certain paraproteins, *e.g.* keratin and amyloid, have been made evident by *Gueft's* investigations (11) Previously *de Brux* (6) has emphasized analogies between amyloidosis and collagenoses. The fluorescence caused by thioflavine S is always enhanced in amyloid according to *Schwartz* (29) In addition to thioflavine fluorescence a positive congo-red staining was found by us in the liver cells with fibrinoid necrosis and also in some cases of periarteritis nodosa (see also 29) Of course, this does not mean that these substances are analogous. However there probably could exist some common compound in these materials.

The investigations by *Novelli* (24) have drawn attention to the fact that a simple staining with haematoxylin-eosin results in a strong golden-yellow fluorescence of several structures (elastic and collagen fibres, hyaline substances, bones, necrotic material) These substances are in fact all more or less autofluorescent, the eosin staining increasing this property to a large extent (dichroic fluorescence) We investigated routinely all our sections. Staining according to van Gieson, *Goldner Kockel, Heidenhain, the* PTAH technique and often also Ladewig staining extinguishes the autofluorescence. Other stains, however like eosin PAS, congo red methylgreen-pyronine and particularly Taft's were often associated with a substantially intensified fluorescence. The latter method was very useful in establishing the presence of isolated necrotic cells which for instance may be difficult to recognize in liver biopsies.

CONCLUSION

On the basis of the results achieved and of the experience gathered we claim that fibrinoid necrosis may occur not only in connective tissue and in vessel walls, but also under certain conditions in parenchymal cells of the liver In the present investigation such necrosis could be observed in the liver cells in cases of toxic, septic and infectious processes and in cases of circulatory disturbances of the organ. Fibrinoid necrosis may also be provoked experimentally *in animals subjected to* chloroform (either by inhalation or subcutaneous administration)

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STRUCTURE OF THE ADRENAL GLANDS IN MICE WITH THE OBESE-HYPERGLYCAEMIC SYNDROME (GENE SYMBOL *ob*)

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Naeser Peter Structure of the adrenal glands in mice with the obese-hyperglycaemic syndrome (gene symbol *ob*) Acta path. microbiol. scand. Sect. A, 83 120–126 1975

Quantitative histological methods were used to study the adrenal glands of obese-hyperglycaemic mice and their lean litter mates of various ages. Five weeks old obese mice had significantly enlarged adrenals, due to increased volumes of the zona fasciculata and a persistent cortical X zone. The enlarged zona fasciculata persisted also in the 5- and 18-month-old obese mice whereas the X-zone had disappeared at these ages. Further the adrenals of 18-month-old obese mice showed deposits of amyloid in the juxtamedullary cortical zone. It is apparent that the adrenal cortical enlargement coincides in time with the appearance of the other manifestations of the obese-hyperglycaemic syndrome. This further emphasizes that also the adrenal cortical enlargement is an integral part of the syndrome and may contribute to the insulin resistance already at an early stage in the development of the syndrome. The more pronounced weight gain of the adrenal glands of the obese mice together with the morphological changes of the adrenal cortex further suggest an increased ACTH stimulation in these animals. The delayed disappearance of the adrenal X zone probably reflects the hypogonadism previously demonstrated in these animals.

Key words Obese-hyperglycaemic syndrome adrenal glands histology

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Previous data indicated a marked enlargement of the adrenal cortex in mice with the obese-hyperglycaemic syndrome (*Marshall et al.* 1957 *Hellerström et al* 1962) This observation agrees well with the recent finding in these animals of both increased concentrations and an enhanced turn-over rate of circulating corticosteroids in these animals (*Naeser* to be published) Furthermore studies of adrenalectomized animals showed that the increased production of corticosteroids contributed to the marked insulin resistance observed in the obese-hyperglycaemic mice (*Naeser* 1973)

In order to evaluate in more detail the significance of hyperadrenocorticism for the development of the obese-hyperglycaemic syndrome, the relationship between the adrenal enlargement and the time of appearance of the various manifestations of the syndrome was studied by quantitative histological analyses of the adrenal cortex. A morphological study of this kind also allows an evaluation

TABLE 1 *Body Weight (g) and Adrenal Weight (mg) of Obese and Lean Mice of Various Ages*

Age	Animals	Body weight (g)	Adrenal weight (mg)
14 days	obese (4)	6.6±0.4	0.67±0.07*
	lean (5)	7.1±0.1	0.63±0.01
18 days	obese (5)	9.4±1.0	0.85±0.14
	lean (4)	7.8±0.4	0.95±0.17
3 weeks	obese (5)	11.4±1.3	2.2±0.1
	lean (5)	9.5±0.6	2.0±0.2
5 weeks	obese (6)	27.1±1.2	4.4±0.3
	lean (5)	21.3±1.0	3.4±0.1
5 months	obese (4)	48.0±1.4	6.5±0.4
	lean (4)	29.1±1.3	3.5±0.1
18 months	obese (4)	41.2±3.8	6.4±0.6
	lean (2)§	29.0 and 32.2	4.1 and 4.2

* Measurement comprising only the left adrenal.

§ In this group of mice only 2 animals survived until 18 months of age.

Note that the adrenal weights in the 14 and 18 days old mice represent only the left adrenal. Mean values ± standard error of the mean (S.E.M.) Numbers of observations within parentheses.

of the extent to which the X-zone of the adrenal cortex contributes to the enlarged adrenal glands.

MATERIAL AND METHODS

Male mice with the obese-hyperglycaemic syndrome (gene symbol *ob*) and male, lean litter mates were used in the present study. The animals belonged to a colony originating from The Jackson Laboratory, Bar Harbor, Maine, USA and bred at the Department of Histology, University of Uppsala, Uppsala, Sweden since 1959 as described previously (*Naeser* 1973).

The age of the animals varied from 10 days to 18 months. In the 10-, 14- and 18-day-old mice it was not possible to identify animals homozygote for the *ob*-gene. To obtain adrenal glands from these animals, a left-sided adrenalectomy was therefore performed on all the mice of a litter. Later the operated animals could easily be classified into two separate groups through the development of visible obesity in the homozygous mice. Other groups of animals, 3 or 5 weeks and 5 or 18 months old, were killed by cervical fracture and both adrenals of these mice were used. All adrenal glands were placed in glass vials containing ice cold 0.9 per cent (w/v) NaCl solution. After careful drainage on a filter paper they were weighed on a torsion balance with a sensitivity of ± 0.05 mg; the error of a single weight determination was ± 5 per cent. After fixation in 10 per cent (v/v) formaldehyde solution and embedding in paraffin, each adrenal was cut into 10 µm thick serial sections. Special care was taken to process the glands uniformly. Adequate differentiation of the cortical zones was obtained after staining in haematoxylin and eosin. In the 18-month-old group, the sections were restained in an alkaline congo red solution and analysed with polarized light for the presence of amyloid (*Puchtler et al.* 1962). All sections were projected onto a drawing paper by the aid of a microscope projector at a total magnification of 200 × and the volumes of the different zones of the cortex and the medulla were calculated separately after planimetry of the projected images (*Kränkó* 1955). A preliminary study showed that it was sufficient to measure every 10th section. By this means the error of a single planimetric determination of the whole gland was found to be ± 1.1 per cent of the mean volume. For the zonae fasciculata and glomerulosa the error was ± 1.1 per cent while it was ± 1.1 per cent and ± 3.2 per cent, respectively, for the X-zone and the medulla.

The correlation between the weight of the adrenal glands and the body weight was tested by linear regression and expressed as the coefficient of correlation (r).

RESULTS

The increase with age of the body and adrenal weights in obese and lean mice are shown in Table 1. Not until the age of 5 weeks were the obese mice significantly heavier than their lean litter mates ($P<0.01$); subsequently they showed the greatest weight difference at the age of 5 months ($P<0.001$).

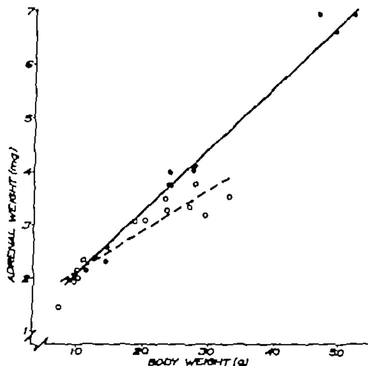


Fig 1 The relation between the weight of the adrenal gland (mg) and the body weight (g) of obese (●) and lean (○) mice 3 and 5 weeks and 5 months of age. The two lines represent the regression lines of obese (——) and lean (– – – –) mice.

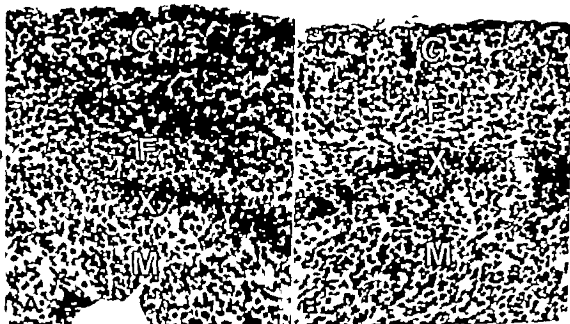


Fig 2 Parts of the adrenal glands of a 14 days old obese (a) and a lean (b) mouse. The X-zone is seen next to the medulla in the lower part of the picture. The X-zone cells have somewhat smaller nuclei and a darker (eosinophilic) cytoplasm. G zona glomerulosa, F zona fasciculata, X X-zone, M medulla. × 700 (Haematoxylin-eosin)

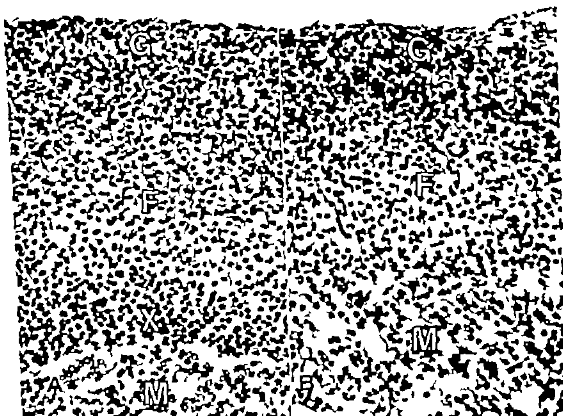


Fig. 3 Parts of the adrenal glands of a 5 weeks old obese (a) and a lean (b) mouse. Note the persisting X-zone and the wide zona fasciculata in the obese mouse. × 700 (Haematoxylin—eosin)

In the oldest age group of obese mice (18 months) neither the body nor adrenal weights were significantly lower than at 5 months. This probably holds true also of the lean litter mates, but unfortunately only two survived 18 months.

The relationship between the weight of the adrenal glands and the body weight is presented in Fig. 1 where the values obtained in animals aged 3 weeks, 5 weeks and 5 months have been used. It applies to obese and lean mice alike that there was a linear relationship with linear correlation coefficients of 0.97 and 0.91 for the obese and lean animals, respectively. In an analysis of variance, the slopes of the regression lines were different in the two groups ($P<0.05$) indicating that the increase in adrenal weight was greater per unit body weight in the obese animals.

Histological examination of the adrenal glands obtained from the 10-day-old animals showed that in the light microscope it was impossible to distinguish the cortex from the medulla. Furthermore, the developing X zone could not be clearly distinguished from the zona fasciculata. For these reasons the adrenals of the 10-day-old animals were not useful for quantitative histological measurements. There were, however, no qualitative differences between the adrenals of the mice homozygous for the *ob*-gene and the lean controls at this age.

In the mice aged 14 days and more it was possible to distinguish two or three separate cortical zones (Figs. 2–4). The most peripheral part of the cortex was the distinct zona glomerulosa. Next to that, the zona fasciculata extended to the border of the X zone in



Fig 4 Parts of the adrenal glands of an 18 months old obese (a) and a lean (b) mouse, × 700 (Haematoxylin-eosin) The pale inner cortical part of the adrenal from the obese mouse (a) represents amyloid and displays birefringence in polarized light (c) × 1100 (alcaline congo red)

the young mice or to the medullary border in the adult mice. The zona reticularis is poorly defined in mice (*Chester Jones* 1948) and was not measured separately On the other hand the inner juxtamedullary X zone was clearly delimited in the age groups 14 days to 3 weeks in the lean animals and up to 5 weeks in the obese mice The cells of this 3–5 cell layer wide zone were more irregularly arranged than those of the zona fasciculata (Fig 2) They were identified by their smaller and darker nucleus and a stronger eosinophilia of the cytoplasm. In the border zone between the medulla and the X-zone cell groups from both parts were often interspersed among each other giving a less distinct morphological boundary In the 18-month-old obese mice the medulla and cortex were separated by a relatively broad, ill-defined zone, characterized by a pale ex

TABLE 2. *Volumes (mm³) of Zonae Glomerulosa, Fasciculata, the X Zone and the Medulla of the Adrenal Glands in Table 1*

Age	Animals	Glomerulosa	Volume (mm³) fasciculata	X-zone	Marrow
14 days	obese (4)*	0.10±0.02	0.08±0.02	0.03±0.01	0.04±0.01
	lean (5)*	0.10±0.01	0.09±0.01	0.03±0.004	0.04±0.01
18 days	obese (5)	0.10±0.01	0.16±0.02	0.03±0.007	0.05±0.01
	lean (4)*	0.12±0.02	0.18±0.04	0.03±0.004	0.06±0.01
3 weeks	obese (5)	0.29±0.02	0.43±0.04	0.09±0.01	0.12±0.01
	lean (5)	0.26±0.07	0.42±0.03	0.08±0.02	0.11±0.01
5 weeks	obese (6)	0.49±0.03	1.13±0.08	0.14±0.01	0.25±0.02
	lean (5)	0.43±0.03	0.86±0.04	absent	0.20±0.01
5 months	obese (4)	0.57±0.03	1.90±0.09	absent	0.41±0.03
	lean (4)	0.41±0.02	0.90±0.07	absent	0.38±0.03
18 months	obese (4)	0.74±0.02	1.69±0.13	absent	0.65±0.09
	lean (2)‡	0.55 and 0.70	1.18 and 1.09	absent	0.41 and 0.50

* Measurement comprising only the left adrenal.
‡ In this group of mice only 2 animals survived until 18 months of age.
The values in the 14 and 18 days old mice represent only the left adrenal. Mean values ± standard error of the mean (S.E.M.)

tracellular homogeneous eosinophilic material. This material stained bright red with congo red, it displayed a green birefringence in polarized light and was interpreted as amyloid. No similar deposits of adrenal amyloid were found in either of the two equally old, lean litter mates.

The data obtained from quantitative histological analyses are presented in Table 2. There were no differences between the two kinds of mice at 14 days, 18 days or 3 weeks of age. By contrast, the 5 weeks old obese mice displayed a significantly larger volume of the zona fasciculata than their lean litter mates ($P<0.01$). Further at this age the X zone was still present in the obese mice, while at 5 months of age the X zone had disappeared. Also at the latter age, the obese animals showed an enlargement of the zona fasciculata ($P<0.001$) and had furthermore a larger zona glomerulosa than the lean litter mates ($P<0.01$). In the 18-month-old obese animals, the zona glomerulosa and zona fasciculata seemed to remain enlarged. In both types of all age groups, relatively large blood sinusoids were present in the medulla. No attempt was made to measure the volume of the medulla cells *per se* and the values presented include also the blood vessels. There was, however no apparent difference between the obese and the lean animals as regards the volume of the medulla plus the blood vessels.

DISCUSSION

It is apparent from the present data that the adrenal cortical enlargement coincides in time with the appearance of the other manifestations of the obese hyperglycaemic syndrome. This further emphasizes that also the adrenal cortical enlargement is an integral part of the syndrome and may contribute to the insulin resistance already early in the development of the syndrome.

In a discussion of the present data, it is notable that *Molne* (1969) showed a marked enlargement of the zona fasciculata in the adrenals of adult mice subjected to prolonged ACTH injections. Furthermore, this treatment caused a significant increase in the nuclear and nucleolar size of the cells, not only the zona fasciculata but also in the zona glomerulosa, suggesting that also the cells of the latter zone may be stimulated functionally by ACTH. On the basis of recent *in vivo* and

in vitro studies of the adrenal function in obese-hyperglycaemic mice (*Naeser* to be published) it has been suggested that the hyperadrenocorticism of these mice is a result of a continuous functional stimulation by ACTH. The present findings support this view

The X zone of the adrenal cortex of mice normally disappears at puberty in the males and at the first pregnancy in females. In the virgin female mouse it cannot be seen after the age of 4–5 months (*Howard Miller* 1927) Administration of testosterone or progesterone also induces degeneration of the X zone (*Howard* 1940 *Holmes & Dickson* 1971) Against this background, the delayed disappearance of the X zone in the obese mice may be associated with their infertility which seems to be due to a low production of sexual hormones (*Hellman et al.* 1963) Although ACTH injections exhibit effects both on zona glomerulosa and zona fasciculata (*Molne* 1969) it is noteworthy that neither stress nor exogenous ACTH administration induce degeneration of the X zone (*Holmes & Dickson* 1971) In fact, the functional role of this particular zone is still a matter of dispute although there is some evidence to suggest that steroids are produced also in the X zone (*Sato* 1968)

The cause of adrenal amyloid deposits mainly in the juxtamedullary zone of the aged obese animals is difficult to evaluate Although amyloidosis in old mice is well known (*Thung* 1957) it remains to be clarified whether the amyloid deposits of the obese-hyperglycaemic mice represent a primary or secondary amyloidosis of the adrenal gland.

In conclusion, the present findings of a more pronounced weight gain of the adrenal glands per unit body weight in the obese mice together with the morphological changes of the adrenal cortex support the view of an increased ACTH stimulation in these mice. Furthermore, the delayed disappearance of the adrenal X zone contributes to the adrenal enlargement and probably reflects the hypogonadism in the obese-hyperglycaemic mice.

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ATYPICAL GERM CELLS IN THE ADJACENT 'NORMAL" TISSUE OF TESTICULAR TUMOURS

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Skakkebæk, N E. Atypical germ cells in the adjacent "normal tissue of testicular tumours. Acta path. microbiol. scand. Sect. A, 83 127–130 1975

Recently a possible intratubular carcinoma-in-situ of the human testis was described. The same histological picture has been found in the residual testicular tissue in 17 out of 22 men with either seminoma, embryonal carcinoma or terato-carcinoma. The hypothesis is put forward that the described cells may represent malignant germ cells from which testicular germ cell tumours of different types may originate.

Key words: Germ cells, atypical testicular tissue testicular tumours.

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Atypical intratubular germ cells were found recently in testicular biopsies from two infertile men (*Skakkebæk* 1972a) Both patients developed embryonal carcinomas within 4½ years. It was suggested that the atypical intratubular germ cells represented a carcinoma-in-situ of the testis (*Skakkebæk* 1972b) In order to test the hypothesis it was decided to examine the non-carcinomatous part of the testis in patients with testicular cancer If the hypothesis was correct, it might be expected, at least in some cases, that atypical germ cells would be found to be located in some of the tubules of the adjacent testicular tissue.

MATERIAL AND METHODS

Histological sections from testicular tissue of 40 patients orchiectomized because of unilateral testicular tumours were studied. In sections from 22 of these patients aged 15 to 58 years (average 37.3 years) preserved tubules were present in residual tissue of the same testis. The number of cases within each type of tumour is shown in Table 1

The testicular tissue was fixed in formalin or Cleland's fixative, sectioned at 4 or 6μ, and stained with iron-haematoxylin or haematoxylin and eosin.

RESULTS

Twenty-two of the 40 preparations contained residual testicular tissue and 17 of these showed tubules with abnormal intratubular germ cells (Fig. 1–4) identical with the previously described atypical germ cells (*Skakkebæk* 1972a and 1972b) while 5 contained apparently normal germ cells only

As compared with normal germ cells, the atypical germ cells were larger The diameter of the nucleus varied and averaged approximately 10 μ (compared with a diameter of



Fig 1 Embryonal carcinoma. Preserved seminiferous tubules (T) located at the edge as the tumour × 115



Fig 2 Same as fig 1 High magnification of a seminiferous tubule Note the atypical germ cells (G) and the apparently normal Sertoli cells (S) × 488.



Fig 3 Same as fig 1 High magnification of a seminiferous tubule Note the occurrence of several layers of intratubular typical germ cells (G) and a cell in mitosis (M) Leydig cells (L) are seen in the interstitial tissue. × 488.



Fig 4 Seminiferous tubule located at the edge of a seminoma. Note the occurrence of typical intratubular germ cells (G) and apparently normal Sertoli cells (S) × 488.

approximately 6 μ in normal spermatogonia). The abnormal cells contained grossly fragmented chromatin and were often dividing (Fig. 3). The abnormal germ cells were generally located in one layer close to the basement membrane, although in some cases several layers of germ cells were found (Fig. 3). As a rule, the atypical cells were the only germ cells present in the actual tubules. In a few cases, however, normal and atypical germ cells were located in the same tubule.

The tubules with atypical germ cells were found in 6 testes with embryonal carcinoma, 3 with terato-carcinoma, one with combined terato-carcinoma and seminoma, and 7 with seminoma (Table 1). In several cases, the tubules with abnormal germ cells were found next to tubules with normal spermatogenesis. In others, all tubules of the residual tissue contained atypical germ cells only in addition to the Sertoli cells which appeared to be normal in all patients (Fig. 2 and 4).

TABLE 1 *Histological Examination of Adjacent Residual Testicular Tissue in 22 Patients with Testicular Tumours*

Type of tumour	No. of patients	
	with atypical germ cells	without atypical germ cells
Embryonal carcinoma	6	0
Terato-carcinoma	3	6
Combined terato-carcinoma and seminoma	1	0
Seminoma	7	4
Reticulosarcoma	0	1

DISCUSSION

The present study supports the assumption that the atypical germ cells described previously (*Skakkebæk* 1972a and 1972b) may represent a carcinoma-in-situ. Seventy-seven per cent of the tumours examined, mainly embryonal carcinomas and seminomas, were accompanied by such atypical intratubular germ cells in the surrounding testicular tissue.

These atypical germ cells are normally not found in the human testis. No such germ cells were reported in quantitative studies of human seminiferous epithelium of fertile men (*Rowley & Heller* 1971, *Skakkebæk & Heller* 1973). Nor could they be demonstrated in normal men in whom spermatogenesis was found experimentally to be impaired (*Heller & Heller 1970*), nor were they found in patients with chromosome abnormalities and severe testicular defects (*Skakkebæk et al.* 1973a and 1973b). However, in testicular biopsies from 4 out of a total of 375 infertile men, the germinal epithelium revealed such atypical germ cells. 3 of these patients developed a testicular tumour (*Nielsen et al. 1974*). The same study also indicated that the atypical cells might be found bilaterally and, ultrastructurally, they had some features in common with prepuberal gonocytes.

It is interesting that the atypical cells were found together with both embryonal carcinoma and seminoma. Co-existence of seminoma and embryonal carcinoma or terato-carcinoma in one testis has been reported repeatedly and this association may have some causal explanation (*Collins & Pugh* 1964, *Dixon & Moore* 1952, *Mostofi* 1973, *Teilum* 1971, *Willis* 1958) although the aetiology is as yet not known. *It is tempting to assume that* the atypical germ cells found in residual testicular tissue of pure tumours of seminoma, embryonal carcinomas and terato-carcinomas may represent malignant germinal cells that may develop into different types of germ cell tumours. This hypothesis is in accordance with the present concept that embryonal carcinomas and teratomas are of germ cell origin, although the actual cells from which these tumours originate have not been demonstrated earlier (*Collins & Pugh* 1964, *Mostofi* 1973, *Teilum* 1971, *Willis* 1958). However, the fact that seminomas may develop *from intratubular* germ cells has been reported by several investigators (*Collins & Pugh* 1964, *Dixon & Moore* 1952, *Mostofi 1973*, *Teilum* 1971, *Willis* 1958).

It is not likely that the atypical cells in

seminiferous tubules in general represent an extension of the tumour along tubules, because the same picture was often found in tubules which were not connected with the tumour besides the ratio between atypical cells and Sertoli cells was rather constant 1 1 Furthermore, the same atypical germ cells were found in the opposite non-carcinomatous testis of 2 patients with seminoma and embryonal carcinoma (*Nielsen et al.* 1974)

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RENAL MORPHOLOGY IN PIGS WITH EXPERIMENTAL HYPERPARATHYROIDISM

Light-Microscopic Findings and Some Functional Aspects

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Boquist, L. & Fåhraeus, B. Renal morphology in pigs with experimental hyperparathyroidism. Light-microscopic findings and some functional aspects. Acta path. microbiol. scand. Sect. A, 83 131–138 1975

Light-microscopic examinations were performed in three groups of pigs: one sham-operated control group, one group subjected to thyroidectomy (TX) and one group injected with parat hormone (PTH) after thyroidectomy (TX + PTH) The kidneys of the sham-operated animals were normal, whereas slight to moderate dilatation of some tubules was seen in the TX group. The most prominent changes in the TX + PTH group were calcium deposition, tubular dilatation and degeneration, inflammation, and hyperaemia. In addition there were hyaline casts, PAS-positive globules and mitotic figures in the tubules. It is suggested that the calcium deposition caused by the PTH administration plays a major role in the development of degeneration in the tubular epithelium, and that these structural lesions are of importance in the development of reduced renal function

Key words Renal morphology experimental hyperparathyroidism.

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The parathyroid glands are involved in the regulation of reabsorption and excretion of calcium by the kidneys (*Epstein* 1968) parat hormone (PTH) enhances the tubular re absorption of calcium (*Peacock et al* 1969 *Massry & Coburn* 1973) In hyperparathyroidism it is well-known that functional and morphological alterations may occur in the kidneys of various species, but the exact relationship between these alterations is not clearly understood. Two different types of primary hyperparathyroidism have been suggested to occur in man one of long duration with normal glomerular filtration rate moderately elevated serum calcium values, hypercalciuria, renal calculi and peptic ulcer another with a more rapid progress, impaired renal function, severe hypercalcaemia, osteitis fibrosa generalisata and nephrocalcinosis the main difference between the two types being the presence or absence of renal impairment (*Hjern* 1974) The renal morphology in spontaneous and experimental hyperparathyroidism was well described by *Anderson* (1939) but disagreement exists as to whether the first structural lesions are localized to the proximal (*Cantarow et al* 1938 *Engfeldt*

et al. 1958, *Caulfield & Schrag* 1964 *Yano et al.* 1965 *Berry* 1970) or distal (*Carone et al.* 1960) tubules. Neither is it established whether the calcifications in the renal parenchyma in hyperparathyroidism are primary (*Learner* 1929 *Schneider et al.* 1960) or secondary (*McJunkin et al.* 1932 *Cantarow et al.* 1938, *Carone et al.* 1960) to degenerative changes in the parenchymal cells.

The present work represents the light microscopic part of our studies in pigs with experimental hyperparathyroidism induced by injection of PTH after thyroidectomy which was carried out in order to eliminate the effects of calcitonin. These studies were undertaken with the aim of obtaining information about the correlation between the development of structural lesions in the kidneys and the impairment of renal function in hyperparathyroidism.

MATERIAL AND METHODS

Animals. The studies were performed in female pigs of Swedish Land race, Swedish Yorkshire race and crosses between these races. In all, 40 pigs were used (*Fåhraeus* 1974) and from 25 of them specimens from the kidneys were taken for morphological examinations, for the present light microscopic study as well as for the subsequent electron-microscopic study (*Boquist & Fåhraeus* 1974). The animals were kept in large cages and at the beginning of the experiments they were 10 to 14 weeks old and weighed 16.5 to 28 kg.

The pigs were given commercially available pig food (Piggfor Aroshygdens Lantmannaförbund, Sala, Sweden). The energy content per daily ration was 3.2 Mcal. The food contained 1 200 I.U. of vitamin D_3, 8.4 g of calcium salts and 6.0 g of phosphates. The animals had free access to drinking water.

Experimental procedures. Three groups of animals were used: one sham-operated control group of 12 pigs (SHAM), one experimental group of 8 pigs subjected to thyroidectomy (TX) and another experimental group of 11 pigs which received parathormone (PTH) injections after thyroidectomy (TX + PTH).

The following experimental procedures were used. At first, central venous catheters were implanted and cystostomy was carried out with ligature and division of the urethra to ensure correct urine sampling. Two to 3 days later thyroidectomy or a sham operation was performed. The TX + PTH group received the first PTH injection on the second day (44 hrs) after the thyroidectomy. PTH was given in subcutaneous injections 3 times a day: 8.00 a.m., 3.00 p.m. and 10.00 p.m. The total daily dose was 25 USP units/kg body weight/day. On the 5th day after the thyroidectomy a biopsy was taken from the left kidney and 3 days later another biopsy was taken from the right kidney. All animals in the TX + PTH group were hypercalcaemic (6–7 mEq/l) (*Fåhraeus* 1974).

The following biopsy technique was used. The animals were anaesthetized with halotane-oxygen-nitrous-oxide on a semi-open system after which the kidneys were exposed through an infracostal transversal incision. The renal biopsies were taken from the lower pole as wedge-resections comprising both cortex and medulla, measuring approximately 4 mm at the base. Then each specimen was immediately divided into two halves: one for light-microscopy and the other for electron-microscopy.

The extirpated thyroid glands were not examined microscopically. The parathyroid glands are in pigs located in the thymus and completely separated from the thyroid (*Iversen* 1917 *Littledike* 1967). The thymus and parathyroid glands were not extirpated and thus not studied microscopically.

Further details about the experimental and operative procedures are given in a parallel study (*Fåhraeus* 1974).

Light-microscopic procedures. The specimens from the left and right kidneys were fixed in 10 per cent formalin. The following light-microscopic stains were used: van Gieson's stain, haematoxylin-eosin, periodic acid—Shiff (PAS), von Kossa's stain, Ladewig's stain, Laidlaw's stain and Sudan Black B.

RESULTS

Sham-operated animals (SHAM). No pathological alterations were found in the kidneys of the control animals.

Animals subjected to thyroidectomy (TX). At the examinations both 5 days (i.e. 78 hrs after the first PTH injection in the TX + PTH group) and 8 days (i.e. 150 hrs after the first PTH injection in the TX + PTH group) after the thyroidectomy the kidneys showed slight to moderate dilatation of some tubules, mainly the proximal ones. Occasional hyaline casts and some desquamated epithelial cells could be found in the tubular lumina. No calcifications or any conspicuous inflammatory cell infiltrates were found.

Animals subjected to thyroidectomy and PTH injections (TX + PTH).

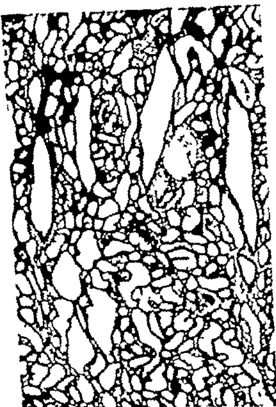


Fig 1 Photomicrograph of porcine kidney showing a few glomeruli and marked, somewhat irregular tubular dilatation. The dilated tubules are empty-looking. TX + PTH 78 hrs. Haematoxylin-eosin (H & E) × 16.



Fig 2 Kidney showing rounded PAS-positive globules in tubular epithelial cells, some of which seem to be degenerated. TX + PTH 78 hrs. PAS, × 100.

Fig 3 Calcium deposits in lumina and epithelial cells of tubules. TX + PTH 78 hrs. von Kossa's stain, × 80.

Fig 4 Inflammatory cells in tubular lumina and interstitium. The lumina are slightly to moderately dilated. TX + PTH; 78 hrs. van Gieson's stain, × 90

Examinations 78 hrs after the first PTH injection The microscopic alterations were essentially similar in all specimens investigated. Most glomeruli showed an ordinary cellularity and normal epithelial and endothelial cells. A few glomeruli were swollen. The capsular epithelium sometimes exhibited proliferations but adhesions were not seen. No calcifications were found in Bowman's space.

A prominent feature was tubular dilatation, affecting all portions of the nephron (Fig. 1) Some segments were dilated up to 6 to 8 times their normal diameter Areas with tubular contraction or atrophy were also found. The tubular epithelium was either normal, degenerated, or proliferating. A few mitotic figures were seen in the tubular epithelium. PAS-positive globules were found in the cytoplasm of some proximal convoluted tubular cells (Fig. 2)

Calcifications were present in the lumina of both dilated and non-dilated tubules (Fig. 3) Calcium deposits were also found in tubular epithelial cells which appeared either normal or degenerated. Obvious degenerative changes were not seen in cells without calcifications. Calcified material occasionally



Fig 5 Portion of kidney showing a marked inflammation of interstitium and tubules. TX + PTH; 78 hrs. H & E, × 40.

Fig 6 Inflammatory reaction and one multinuclear giant cell in interstitium. TX + PTH 78 hrs. van Gieson's stain, × 110.

seemed to be released from degenerating epithelial cells. The size, shape and density of the deposits varied some showed a concentric lamellation. Occasionally calcium precipitations seemed to cause obstruction of the tubular lumina, leading to dilatation of the tubules proximally Sometimes it was difficult to discern whether the calcium deposits were localized to the lumina or epithelial cells of the tubules Due to degenerative changes in the tubular epithelium it could also be difficult to clarify in which portion of the tubular system the calcium deposits occurred. It seemed, however as if they could be situated in all portions of the nephron and in the collecting tubules, although most deposits appeared to be local ·ed to the proximal con voluted tubules.

Dilated and nor ¹¹ ted tubular lumina were also seen conta g hyaline casts, amorphous secretions, desquamated epithelial cells, erythrocytes and inflammatory cells, mainly granulocytes (Fig. 4)

Slight to moderate hyperaemia was occasionally noted. No obvious degenerative changes were seen in the vessels.

The interstitial tissue exhibited localized or diffuse inflammatory infiltrates (Fig. 5) consisting of granulocytes, lymphocytes, plas cells and histiocytes with a tendency to pe tubular and perivascular arrangement. A f multinuclear giant cells, apparently of fore body type were found among the inflamm tory cells (Fig 6) No epithelioid cells we observed. Calcium deposits unequivoca localized to the interstitial tissue were n recorded.

In the renal pelves the epithelium was nc mal The subepithelial tissue was oft oedematous and infiltrated by inflammato

Fig 7 View of kidney showing marked irregular dilatation of tubules. TX + PTH 150 hrs. H & E, × 16.

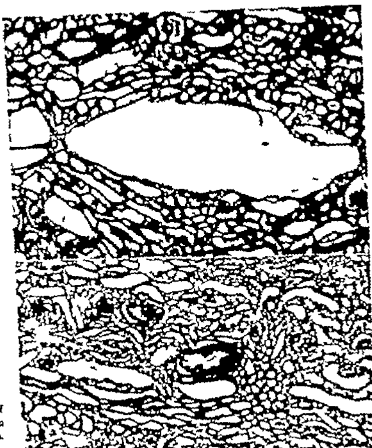


Fig 8 Kidney showing streaks of tubular dilatation and calcium deposits. TX + PTH 150 hrs. von Kossa stain, × 15.

cells. No changes were found in the renal capsule

There was often a tendency to patchy distribution of the morphological changes. Radiating streaks of calcifications and/or dilated tubules could be seen in the renal parenchyma. Portions of condensed renal parenchyma with atrophic tubuli were found between areas with tubular dilatation.

Examinations 150 hrs after the first PTH injection The histopathological changes were more marked than after 78 hrs, especially as to tubular dilatation and calcifications (Fig. 7) There was also a more obvious tendency to focal accumulation of the morphological changes with the appearance of radiating streaks of calcifications and/or dilated tubules (Fig. 8) No qualitative differences were, however observed between the histopathological changes at the two observation times.

DISCUSSION

As early as 78 hrs after the first PTH injection to the thyroidectomized pigs, clear morphological alterations were found in the renal parenchyma. These alterations were more advanced after 150 hrs, which implies that a rapid progressive increase in the renal lesions took place under the influence of PTH. The most conspicuous alterations were calcium deposition and tubular dilatation, which is consistent with the findings in other studies on experimental hyperparathyroidism (*Cantarow et al* 1938 *Carone et al.* 1960) Similar changes are also known to occur in spontaneous hyperparathyroidism in man (*Albright et al.* 1934 *Anderson* 1939 *Hellstrom & Ivemark* 1962)

The fact that calcifications occurred in the kidneys of the animals of the TX + PTH

group but were absent from those of the SHAM and TX groups, indicates that the deposition of calcium was caused directly or indirectly by the injected PTH and the concomitant hypercalcaemia. Hypercalcaemia alone does not seem to give any persisting damage in the human organism although acute symptoms may occur (*Reed* 1934, *Fåhraeus et al.* 1973). The excretion of calcium has been found to be lower in patients with primary hyperparathyroidism than in those with other disorders with associated hypercalcaemia (*Transböl et al.* 1970).

McJunkin et al. (1932) and *Cantarow et al.* (1938) advocated that degenerative epithelial changes preceded the development of calcifications in the kidneys. Degenerative changes clearly preceding the development of calcifications were not observed in our study. Nor were such changes found by *Schneider et al.* (1960). The inflammatory reaction and the giant-cells are assumed to have appeared secondarily to calcium deposition and degenerative cellular changes.

As tubular dilatations sometimes were seen proximally to calcified tubular casts it is believed that obstruction caused by calcific deposits plays a pathogenetic role in the development of the dilatations. Dilatations of the tubular system were also found in portions of renal parenchyma with only sparse occurrence of tubular casts and in kidneys without calcium deposits, as was the case in the TX group. Thus, it appears that also factors other than tubular obstruction by calcific casts participated in the development of the tubular dilatation. Focal internal hydronephrosis caused by tubular obstruction has been observed also in experimental magnesium-deficiency (*Schneeberger & Morrison* 1965).

It is not known whether the PAS-positive globules, which were observed in some tubular epithelial cells, were caused by the PTH administration or whether PTH had any influence on the development of the hyaline casts. Such casts ha[illegible] been suggested to serve as a mucoprote[illegible] [illegible]atrix for the precipitation of calcium sal[illegible] *Boyce et al.* 1954) and in a study of the [illegible]t of parathyroid extract, *Engel* (1952) proposed that the casts in the tubular lumina contained mucoprotein which had reached the kidneys due to a PTH induced release into the blood of mucoprotein residues of the ground substance of bone. *Engel* (1952) also found that the hyaline casts disappeared after cessation of the administration of parathyroid extract, whereas calcific casts remained fixed in the tubules.

The microscopic evidence in this study of hyperaemia in the kidneys of the TX + PTH group is in agreement with the findings that PTH increases the renal blood flow (*Charbon* 1968, *Charbon et al.* 1968, *Charbon* 1969) and that there is a relative renal hyperaemia in patients before operation for hyperparathyroidism which disappears postoperatively (*Edwall* 1958).

In the functional studies of the TX + PTH group (*Fåhraeus* 1974) there was a significant decrease in all clearance values as well as in TRP which is consistent with the findings in other experimental (*Engfeldt et al.* 1962) and clinical (*Björnboe et al.* 1952, *Edwall* 1958, *Hellström & Ivemark* 1962, *Ohlsson* 1970) studies of hyperparathyroidism. Cl_{PAH} and Tm_{PAH} were used to measure tubular function despite the existence of variations in the glomerular filtration. The cause was that these young pigs did not tolerate the dehydration necessary for the determination of the maximum urinary concentration capacity (*Fåhraeus & Lorentzon* 1974). The elevation of the serum calcium level from about 5 mEq/l to 6–7 mEq/l shows that the PTH administration was really effective in inducing a hyperparathyroid state. As a result of the PTH administration there was a significant decrease in the glomerular filtration rate, the renal plasma flow and the transfer maximum of PAH. Changes in the glomerular filtration rate can be caused by variations in the renal blood flow and the blood pressure, and probably also by changes in the intratubular pressure. In this study no structural lesions were found in the glomeruli. However, the casts and dilatations observed in the tubules might have been associated with an increase in the intratubular pressure, which secondarily

might have affected the glomerular filtration rate

PAH is secreted by the epithelial cells of the proximal tubules. Degenerative changes and calcifications, as those observed in this study may very well affect the secretory capacity of these cells and cause a decrease in Tm_{PAH} and Cl_{PAH}

The functional part (*Fåhraeus* 1974) of this study on experimental hyperparathyroidism in pigs disclosed that thyroidectomy was followed by a reduction in the renal function measured by clearance determinations. An even more marked reduction in the renal function was recorded when thyroidectomy was combined with PTH administration. The cause of the functional impairment in animals subjected to thyroidectomy is not clearly known. The morphological alterations in the kidneys of these animals were rather slight. In the TX + PTH group, on the other hand, there were early appearing marked structural changes in the kidneys with signs of cellular degeneration, tubular obstruction and dilatation, and inflammation. There was also evidence of a progressive increase in the structural lesions during the presently used observation period. The mitotic activity observed in the tubular epithelium was apparently not of sufficient magnitude to compensate for the loss of renal parenchymal cells. We believe that the structural changes played a prominent role in the development of the decrease in the renal function. Thus we do not believe that the morphological alterations merely represented secondary changes in kidneys with already established functional impairment.

A reduction in the renal function coupled with morphological lesions has been observed also in experimental hyperparathyroidism in dogs (*Epstein et al* 1959 *Carone et al* 1960 *Engfeldt et al* 1962) and rats (*Engfeldt et al.* 1958) *Engfeldt et al* (1962) recorded an increase in the reduced clearance values after the cessation of PTH administration, but there was no complete recovery which was thought to be due to permanent structural changes in the kidney *Baker & Howard* 1936 found that the renal lesions in patients with hyperparathyroidism could be halted if the disease was treated, but improvement in the renal function was achieved only in early cases. Later investigations have confirmed this finding (*Edwall* 1958, *Hellström & Ivemark* 1962 *Ohlsson* 1970)

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RENAL MORPHOLOGY IN PIGS WITH EXPERIMENTAL HYPERPARATHYROIDISM

Ultrastructural Findings

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Ultrastructural examinations were performed in three groups of pigs one sham-operated control group one group subjected to thyroidectomy and another group injected with parathormone after thyroidectomy (TX + PTH) Attention was mainly paid to the localization and structural details of the calcium deposits which were found in the TX + PTH group 78 and 130 hours after the first PTH injection. Calcium deposits were found in the epithelial cells and lumina of the tubular system, especially in the proximal convoluted tubules. It is suggested that calcium first is deposited in mitochondria and cytoplasmic vacuoles and that larger calcific bodies, occasionally with concentric lamellation, are formed by coalescence of small calcific particles and that the calcium deposits induce degenerative changes in the tubular epithelium which may lead to discharge of calcific material into the lumina.

Key words Renal morphology experimental hyperparathyroidism, ultrastructure, pigs.

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In a preceding light microscopic study we examined the kidneys of one group of pigs subjected to thyroidectomy and of another group of pigs with hyperparathyroidism induced by injection of parathormone (PTH) after thyroidectomy (*Boquist & Fåhraeus* 1974 Only slight structural changes were observed in the former group whereas marked lesions were found in the latter group calcium deposition, dilatation and degeneration of tubules interstitial inflammation and hyperaemia were the most conspicuous alterations. Although calcareous deposits were evident already at the first examination 78 hours after the first PTH injection, their exact localization and configuration were difficult to clarify by light-microscopy The present electron-microscopic study was undertaken with the aim of obtaining further information about the localization and fine structural details of these deposits and their morphogenesis.

MATERIAL AND METHODS

Information about the animals, the feeding and the experimental procedures are given in the preceding paper (*Boquist & Fåhraeus* 1974) Three groups of animals were used 12 sham-operated

pigs, 8 pigs subjected to thyroidectomy and 11 pigs receiving PTH injections after thyroidectomy

Specimens were taken from both kidneys 78 and 150 hours after the first injection of PTH and were fixed in 2.5 per cent glutaraldehyde in 0.34 M Veronal acetate buffer adjusted to pH 7.4 followed by postfixation in 1 per cent osmium tetroxide in the same buffer. After fixation, the specimens were rinsed, dehydrated and embedded in Epon 812. The sections were cut on an Ultrotome III and thick sections stained with toluidine blue were used for light-microscopic identification of suitable areas for the thin sections. After staining with uranyl acetate and lead citrate the sections were examined in a Siemens Elmiscope 101.

RESULTS

The electron-microscopic examinations were mainly directed to the occurrence and morphological details of structures believed to represent calcium deposits.

Sham-operated animals (SHAM). The renal parenchyma was normal. No calcifications were recorded.

Animals subjected to thyroidectomy (TX). No conspicuous ultrastructural changes were observed in the renal parenchyma. Structures which could represent calcifications were not observed.

Animals subjected to thyroidectomy and PTH injections (TX + PTH). The ultrastructural examinations were mainly directed to areas of renal parenchyma which in the light-microscope had been found to possess calcium deposits. Only slight individual changes were observed among the animals. The description below refers to both observation times, 78 and 150 hours after PTH injection, respectively. If findings at the two observation times differed, this will be mentioned in the text.

The light-microscopic findings of dilatation of the tubules and degenerative changes in the tubular epithelium, and the essentially interstitial inflammation were verified in the electron-microscopic examination.

Structures which were interpreted as calcium deposits were found in the tubular epithelial cells, mainly in those of the proximal convolution but to a lesser extent also in other tubular epithelial cells of the nephron and the collecting tubules. Similar structures were frequently found in the tubular lumina, but at the presently used observation times such structures were not clearly observed in the basal laminae, interstitium, glomeruli or vessels.

The mitochondria, especially those of the proximal convolution, were often swollen with partly or completely destroyed cristae and external membranes. Other mitochondria had an atypical configuration and were disintegrating. Still other mitochondria were normal. Lipoid bodies were seen in cells with or without mitochondrial alterations. The matrix of many bodies, probably of mitochondrial nature, was finely granular and contained moderately electron dense amorphous masses with indistinct boundaries (Fig. 1). In the inner compartment of these bodies, within, or occasionally outside the amorphous masses, there were small needle-like electron dense particles. Small electron dense granular particles, sometimes with an annular arrangement, were also observed in these bodies (Fig. 2). The needle-like and granular particles were usually localized outside and close to the cristae. They were never seen inside the cristal membranes. Small electron dense particles were also seen seemingly lying free in the cytoplasmic ground substance, without any relationship to mitochondria (Fig. 3).

Rounded electron dense bodies of varying

Fig. 1. Part of a tubular epithelial cell showing distended mitochondria with partly disrupted cristae and external membranes and a content of amorphous moderately dense masses and small dense particles (arrows). Normal mitochondria (M) and a lipoid body (L) are also seen. TX + PTH; 78 hrs. × 11 000.

Fig. 2. Part of another tubular cell with mitochondria containing amorphous masses and small groups of granular dense particles (arrows). Other mitochondria are normal (M) or swollen (S). TX + PTH. 78 hrs. × 11,000.

Fig. 3. Part of a tubular cell showing a cytoplasmic collection of needle-like electron dense particles (C). TX + PTH; 78 hrs. × 15,000.

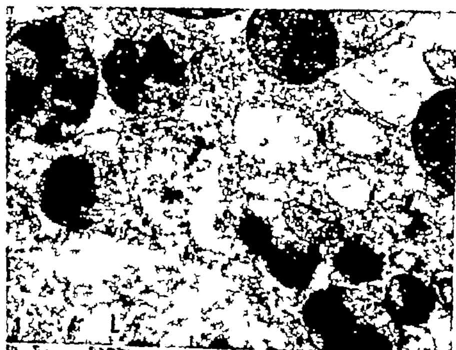
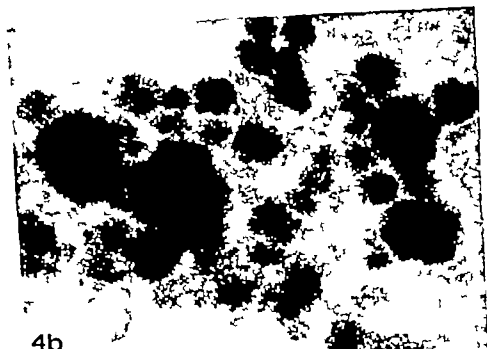

L



Fig. 41 A detail from the cytoplasm of tubular cell showing electron lucent vacuoles with aggregates of rounded dense bodies of somewhat varying size. Membranous structures (m) are seen at the periphery of a large vacuol. Close the vacuoles and mitochondria there are bundles of filaments. TX + PTH: 78 hrs. × 12,000.



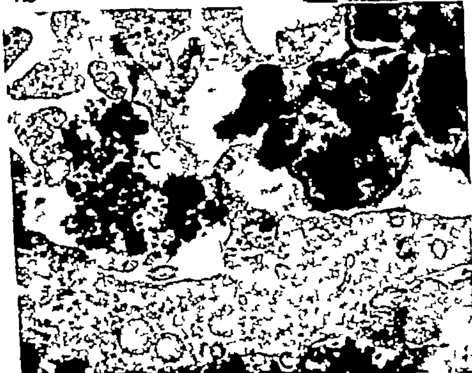


Fig. 4B. Higher magnification of portion of Fig. 4A demonstrating that the rounded bodies are composed of granular subunits, occasionally in annular arrangement (arrow) TX + PTH 78 hrs. × 37,000.

Fig. 5. Part of tubular epithelial cell with cytoplasmic vacuoles containing bodies similar to those in Fig. 4 (C) and large irregular body (upper right) with a trilamellar "shell" and moderately dense "matrix" with bodies similar to those in the other vacuoles (C) TX + PTH 150 hrs. × 9 000

size and with slightly irregular contour were seen in electron lucent cytoplasmic vacuoles (Fig 4A) These bodies were composed of small dense granular subunits, occasionally *exhibiting a tendency to annular arrangement* (Fig 4B) Some vacuoles also contained membranous structures. Bundles of filaments in a more or a less parallel arrangement were seen in the cytoplasm, often close to or in direct contact with the mitochondria (Fig 4A)

The cytoplasmic vacuoles *also* contained larger bodies with a rather smooth outline and an irregular shape (Fig 5) These bodies enclosed small rounded bodies of a type simi lar to that described above and possessed a finely granular or fibrillar matrix of moderate electron opacity At the periphery there was a "shell" composed of parallell finely granular lamellae of varying electron opacity Discontinuities could be seen in this "shell"

Large irregular bodies were also seen, which had a multi layered concentric appearance with lamellae of alternately high and low density (Fig 6) The outline was often slightly irregular and sometimes undu lating Centrally there was often a "nucleus" of high density occasionally with irregular areas of lower density A rim of small granular components of high density was seen on the surface of these bodies. Occasional small or large polypoid projections with a finely granular matrix of moderate electron opacity were seen to protrude from these large bodies. Membrane bound electron lucent bodies were also found, which contained amorphous masses *and membrane-like structures composed* of small granular subunits (Fig 7) They oc curred in cytoplasmic vacuoles which sometimes also contained amorphous masses of moderate electron density

Other myelin like cytoplasmic bodies were composed of closely applied parallel membranes of varying density The membranes encompassed areas that were either electron lucent or electron dense or an area of appa rently normal cytoplasmic ground substance (Fig. 8)

Tubular lumina with light microscopic evidence of calcifications were ultrastruc turally found to contain remnants of disinte grated cells, and structures, apparently representing swollen mitochondria with disrupted *cristae and a content of* needle-shaped or finely granular particles. There were also large electron lucent or moderately dense vacuolar bodies which contained similar par ticles and also small vesicles of varying size (Fig 9) Lipoid bodies and normal mitochondria were observed in the tubular lumina. *Small electron dense particles and large* dense bodies were also seen, lying free in the lumina.

Epithelial cells containing the various cytoplasmic inclusions described above, often ex hibited degenerative changes of varying severity Some cells were disintegrating and their content seemed to be discharged into the lumina. No marked degenerative changes were seen in epithelial cells without these kinds of cytoplasmic inclusions.

The small needle shaped and granular par ticles and the somewhat larger rounded dense bodies were found at both observation times, whereas the large bodies with a lamellar "shell" the large concentric bodies, and the mitotic figures were found mainly or exclusively 150 hours after the first PTH injection. The large membrane-bound electron lucent bodies, the myelin-like bodies and the contents of the tubular lumina described above were recorded at both observation times, but the intraluminal deposits were more extensive at the last observation time.

Fig 6 A cytoplasmic vacuol in a tubular epithelial cell. The vacuole contains large bodies with finely granular concentric lamellae of varying density and an electron-dense central portion with some irregular electron-lucent areas. The bodies are connected to each other and one of them has a polypoid projection. Amorphous masses of low density are also seen in the vacuoles TX + PTH; 150 hrs. × 17,000.




A
A
7
8



Fig 9 Portion of a tubular lumen showing microvilli (lower left) from an epithelial cell and large membrane-bound electron lucent bodies (V) which contain small dense particles and vesicles. An unaffected mitochondrion is also seen (arrow) TX + PTH 150 hrs. × 9,000.

DISCUSSION

Cells with light microscopic evidence of calcium deposition were ultrastructurally found to contain cytoplasmic inclusions of various kinds small needle-shaped or granular particles, somewhat larger and rounded bodies composed of small granular subunits, large bodies with a "shell" of peripheral lamellae, concentric lamellar bodies, membrane-bound electron lucent bodies with granular membranous contents, and myelin-like bodies. The small needle-shaped and granular intramitochondrial particles are similar to those found by *Caulfield & Schrag* (1964) in the proximal renal tubules of mice injected with PTH. These authors stated that the needle-shaped particles had the diffraction pattern of apatite. The large lamellar bodies found in the present study resemble the calcium-laden cytoplasmic bodies observed at extrarenal sites in non-parathyroid diseases, *e.g.* malakoplakia of the skin (*Price et al* 1973) which also show a diffraction pattern consistent with that of apatite. Cytoplasmic inclusions of the kinds described above were not found in the sham-operated pigs or in the pigs subjected to thyroidectomy i.e in animals without any calcareous deposits in the kidneys. Against this background it seems obvious that the cytoplasmic inclusions illustrated in Figs. 1 to 6 represent calcium deposition in the tubular epithelial cells. As the membrane bound electron lucent bodies (Fig. 7) also

Fig 7 Part of the cytoplasm of a tubular epithelial cell showing rather big vacuoles. These contain moderately dense amorphous masses (A) some membrane-bound electron lucent bodies with contents of amorphous material and some annular membrane-like structures composed of small granular subunits. Bundles of filaments (F) are also seen close to the vacuoles TX + PTH 78 hrs. × 9,000.

Fig 8 Part of a tubular epithelial cell with a myelin-like cytoplasmic body showing closely applied, parallel membranes which surround areas of apparently normal cytoplasm. TX + PTH 150 hrs. × 20,000.

10*

possessed small granular subunits, it is probable that also these bodies contained calcium. The amorphous masses seen in some mitochondria (Fig 1) might also represent calcium, possibly in an amorphous state.

Degenerative changes were often seen in mitochondria containing calcific particles and it is possible that the needle-shaped particles sometimes occurring in the cytoplasmic ground substance had been discharged from disintegrated mitochondria. The rounded electron dense bodies occurring in cytoplasmic vacuoles (Fig 4A and B) might also have originated from mitochondria. The membranes occasionally found in these vacuoles might represent remnants of mitochondria. However in the absence of obvious signs of delivery of calcific material from mitochondria to vacuoles, the possibility also remains that the rounded calcific bodies had been primarily formed in the vacuoles without any intermediate mitochondrial stage. Formation of calcific particles in vacuoles of unicellular organisms has been reported (*Pautard* 1970)

The large irregular bodies with a lamellar periphery contained bodies identical to the rounded bodies seen in some cytoplasmic vacuoles (Fig 5) These large bodies are believed to be formed by coalescence of such rounded bodies. The bundles of fibres observed in the cytoplasm around the vacuoles may also take part in the formation of these large bodies, since fibrillar material occurred in their "matrix" The peripheral lamellae of these large bodies are believed to represent an early stage in the development of multilayered concentric bodies (Fig 6) The formation of the concentric rings may be related to the Liesegang phenomenon of crystal growth in gels (*Liesegang* 1926 *Alexander & Johnson* 1949) The suggested development of calcific bodies does not have to proceed until the formation of concentric bodies, but may stop at any suggested stage in this development.

Myelin-like bodies (Fig 8) are usually considered to be associated with degenerative lesions. These bodies may be related to the existing hypercalcaemia and calcium deposition, since they were found in animals injected with PTH but were absent from the kidneys of sham-operated pigs and animals subjected to thyroidectomy alone.

The degenerative changes occurring in mitochondria or whole cells of the PTH injected animals but not in the other groups of animals are believed to have been caused directly or indirectly by the calcareous deposits. An increase in mitochondrial calcium has been suggested to precede (*Buffa & Colajanni* 1953) or be a very early manifestation (*Judah et al.* 1964) of cell necrosis.

At least some of the calcific material observed in the tubular lumina seemed to have been discharged from disintegrating epithelial cells. This assumption is supported by the findings of degenerated mitochondria and also some apparently normal mitochondria in the lumina.

In contrast to the observations in rats (*Engfeldt et al* 1958) mice and dogs (*Yano et al.* 1965 *Berry* 1970) we found no structures which unequivocally represented calcium deposition in basement membranes. *Yano et al* (1965) stated that the deposition in the basement membranes represents the first evidence of nephrocalcinosis in experimentally induced hyperparathyroidism. This could not be verified in the present study

The intramitochondrial particles found in our animals are believed to be the first evidence of deposition of calcium in the tubular epithelium. Such intramitochondrial deposition of calcium is not unexpected in regard of the well-known tendency of divalent cations to be taken up by mitochondria in which deposits may be seen as electron dense structures (*Lehninger* 1965) The mitochondrial uptake of calcium is a very rapid process (*Hackenbrock & Caplan* 1969) At massive loading of mitochondria with excess calcium there is a tendency for swelling unfolding of cristae and separation and/or loss of the outer membrane (*Lehninger et al.* 1967) Kidney mitochondria may increase their calcium content rapidly more than ten times their original amount in uranium intoxica-

tion (*Carafoli et al.* 1971) The rounded calcific bodies which were found in vacuoles may also represent an early manifestation of intracellular calcium deposition, possibly occurring secondarily to calcium accumulation in mitochondria.

As far as can be concluded from our results it is obvious that calcium deposition in the kidneys occurs rapidly under the influence of a high concentration of circulating PTH with concomitant hypercalcaemia. It is also evident that the early intracellular deposition of calcium mainly although not exclusively is localized to the proximal convoluted tubules the part of the nephron known to be influenced by PTH (*Gekle* 1971) A modulating effect of PTH upon the ion movements in mitochondria has been suggested by *Lehninger et al.* (1967)

Although it can not be excluded that the degenerative changes in the parenchymal cells represents artifacts we believe that at least some of these changes appeared secondarily to the calcium deposition and lead to a subsequent discharge of calcific material into the lumina with eventual tubular obstruction. Such obstruction might affect the tubules proximally and cause a further decrease of the available functioning tubular parenchyma.

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THE EFFECT OF RAUSCHER AND MOLONEY LEUKAEMIA VIRUS ON AMYLOID DEVELOPMENT IN CASEIN-TREATED CBA MICE

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Infection of adult CBA mice with Rauscher or Moloney leukaemia virus concomitantly with caseination significantly accelerated spleen amyloid development in irradiated, bone-marrow protected mice, but had no effect on untreated, adult thymectomized or thymectomized irradiated mice. Spleen tissue of mice infected with Moloney virus had the highest titre in the mice with accelerated amyloid development.

Key words: Leukaemia virus, Rauscher Moloney amyloid casein-treated CBA mice.

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It has previously been demonstrated that murine leukaemia virus may interfere with amyloid development in caseinated intact adult C3H mice (*Ebbesen* 1973). In view of the importance of cellular immune reactions for leukaemia (*Dent et al.* 1965) and amyloid (*Cathcart et al.* 1971) development, we have investigated both phenomena in mice with experimentally modified thymus-derived (T) cell pools.

MATERIAL AND METHODS

Virus. Rauscher leukaemia virus (*Rauscher* 1962) and Moloney leukaemia virus (*Moloney* 1960) were harvested from culture medium of infected secondary BALB/c embryo fibroblast cultures and titered by the in vitro XC-test (*Rowe et al.*, 1970) and by intraperitoneal inoculation of adult CBA mice with recording of survival time. To determine the virus concentration in spleens, the organ was removed, immediately frozen at −70 C, and later made into a 10 per cent extract which was tested for leukaemia virus by the XC-test.

Mice. CBA/Lac male mice were used throughout and were divided into four groups with different T-cell pools (*Davies et al.* 1966 *Doenhoff et al.* 1970 *Davies et al.*, 1971)

A) Unoperated control mice. B) Mice thymectomized at 8 weeks of age. C) Adult thymectomized animals given 850r total body irradiation and 5×10^6 syngeneic bone-marrow cells (T-cell deprived mice). D) Non-thymectomized irradiated

mice given 5×10^{6} syngeneic bone-marrow cells (T-cell reconstituted mice)

Casein. A 5 per cent solution in 0.25 per cent sodium hydroxide was used.

Experimental procedure. Eight weeks after irradiation and bone-marrow reconstitution, casein was first administered to the mice. Six subcutaneous injections a week were given for 8 weeks. Seven control mice in each group were treated with saline alone (see Table 1) In one experiment 1.5×10^{4} XC-units *Rauscher* virus and, in another experiment, 1.5×10^{4} XC-units Moloney virus were given intraperitoneally once, simultaneously with initiation of casein treatment. Pre testing showed this dose *to kill about half of the* mice in six months. Controls received one inoculation of culture medium from uninfected BALB/ embryo fibroblasts.

Two days after the last casein inoculation, the animals were killed Lung, liver, kidney spleen, peripheral lymph nodes, thymus, and thyroid gland were taken for histology and stained with periodic acid-Schiff (PAS) and alkaline Congo red. Amyloid was identified by its morphology and by its bi-refringence with Congo red under crossed polars. The degree of spleen amyloidosis was calculated on PAS-stained sections and graded from 0 to 6 (three indicating an unbroken ring of amyloid around spleen follicles) (*Christensen et al.*, 1959) Statistical calculations were by the Student *T-test. The content of leukaemia virus* in the spleens at time of autopsy was determined as previously described. T obtain a measure of the content of bone-marrow cells capable of growing in plastic bottles at time of autopsy the marrow cells were flushed from 1 cm of both femoral shafts with Minimum Essential Medium (Eagle) with Hank Balanced Salt Solution pH 7.4 and 10 per cent foetal calf serum (MEM) The cells from each experimental group were pooled and dispersed into a monocellular suspension in the cold. Three 30 ml Falcon plastic bottles were *seeded with 1.. × 10 nucleated cells from* each group and kept at 37 C in 20 ml MEM. Medium was changed the following day and the fixed cells counted on day 7

RESULTS

Amyloid, if present, was always found in the spleen. Grades 4 and 5 were often accompanied by liver amyloidosis. Other organs were free of amyloid. No amyloid was present in mice not given casein.

No statistically significant difference in the degree of amyloid in A) uninfected unoperated, B) thymectomized, C) T-cell deprived mice and D) T-cell reconstituted mice in the two experiments carried out (Table 1) was found. Both Rauscher and Moloney leukaemia virus enhanced amyloid formation but only in the T-cell reconstituted animals (D) Rauscher and Moloney viruses did not differ significantly with respect to their influence on amyloidogenesis.

Small leukaemic infiltrates were present *in* spleen and liver of a single mouse in some infected groups. However several animals in the Rauscher virus infected T-cell deprived group had small infiltrates. Mice with leukaemia infiltrates had neither more nor less amyloid than other mice in their group. No statistically significant difference in leucocyte counts in the various experimental groups was found.

Leukaemia virus with a low titre was recovered from thymectomized-irradiated caseinated mice and from irradiated saline treated mice. Caseinated irradiated mice had 10^{3} times more virus per gram spleen than saline treated irradiated mice.

Seven-day-cultures established with bone marrow from caseinated mice contained 50 to 100 per cent more fixed cells than cultures established from saline treated mice, if the cells originated from A) unoperated, B) thymectomized and C) T-cell deprived mice. However bone-marrow cells from T-cell reconstituted mice (group D) did not show this effect. When cultures from caseinated mice of group A, B and C only were considered statistically cultures from caseinated mice contained *significantly more cells than* cultures from saline treated controls $0.005 < p < 0.01$ When all cultures from caseinated mice (Groups A, B, C and D) were compared with cultures from all groups of saline treated mice with regard to the number of fixed cells we got $0.01 < p < 0.02$. Bone-marrow cell cultures derived from virus-infected caseinated mice differed less than 20 per cent from cultures derived from caseinated non-infected mice with regard to number of cells surviving after one week. Peroxidase staining (Endicott and Gump, 1947) showed the presence of polymorphonuclear granulocytes and monocytes in all cultures.

TABLE 1 *Influence of Infection with Rauscher (R) or Moloney (M) Leukaemia Viru*

Group	Pretreatment		No. of mice	Spleen		
				No. with amyloid	Mean amyloid	P
A	None	casein	6	6	3.0	NS
		casein + R	10	10	3.6	
		NaCl	3	0	0	
		NaCl + R	4	0	0	
B	Thymectomy	casein	6	5	2.4	NS
		casein + R	8	8	3.3	
		NaCl	3	0	0	
		NaCl + R	3	0	0	
C	Thymectomy + irradiation + BM cells (T-cell deprived)	casein	6	5	3.2	NS
		casein + R	7	7	2.9	
		NaCl	3	0	0	
		NaCl + R	3	0	0	
D	Irradiation + BM cells (T-cell reconstituted)	casein	6	6	3.5	<0.001
		casein + R	8	8	4.9	
		NaCl	3	0	0	
		NaCl + R	3	0	0	
A	None	casein	12	12	3.1	NS
		casein + M	1	12	3.3	
		NaCl	3	0		
		NaCl + M	3	0		
B	Thymectomy	casein	6	6	3.2	NS
		casein + M	8	8	3.4	
		NaCl	3	0		
		NaCl + M	3	0		
C	Thymectomy + irradiation + BM cells (T-cell deprived)	casein	6	6	2.5	NS
		casein + M	8	8	3.7	
		NaCl	3	0		
		NaCl + M	3	0		
D	Irradiation + BM cells (T-cell reconstituted)	casein	6	6	3.0	<0.01
		casein + M	8	8	4.5	
		NaCl	3	0		
		NaCl + M	3	0		

DISCUSSION

Cellular leukaemogenic extracts of some murine plasma cell leukaemias and reticulosarcomas type B have been shown to have an amyloid promoting effect in syngeneic mice (*Ebbesen & Rask Nielsen* 1967).

The first work on casein-induced amyloid in mice infected with isolated murine leukaemia virus indicated a possible effect, although the level of significance was low (*Ebbesen* 1973) in the present work a significant amyloid-promoting effect of two leukaemia viruses was demonstrated in irradiated T-cell reconstituted mice. Since the virus was harvested from BALB/c mouse cultures which have been found free of common pathogens (*Ebbesen et al.* 1973) interference by other viral or non-viral microorganisms from tissue culture sources is unlikely

n. Amyloid Development in Casein Treated CBA Mice with Modified T-Cell Populations

Number of mice with histologically verified leukaemic infiltrates	Mean number of fused cells $\times 10^8$ in vitro 7 days after seeding 1.2×10 nucleated bone-marrow cells (six bottles in each group)	XC-units leukaemia virus per gram spleen tissue (mean of 3 determinations)
1		
1		
1		
5		
1		
1		
	1.72	0
	1.05	0
	1.72	0
	1.16	0
	1.50	$2 \times 10^{[illegible]}$
	0.78	0
	0.87	$3 \times 10^{[illegible]}$
1	0.75	$1 \times 10^{[illegible]}$

Virus induced malignant transformation of spleen cells appeared unrelated to amyloid development as judged from histology. The viral antigens add to the antigen load on the caseinated animals and perhaps in that way enhance amyloid formation. However, the immune reactions of adult mice decrease the titre of Moloney virus (*Klein & Klein* 1964, 1965) and we found the highest virus titre to be present in the spleens with the most amyloid. A secondary effect of virus growth in the amyloid producing cells precipitation of viral protein or viral transfer of genetic information directly relevant to amyloid production therefore seems more likely explanations of the amyloid inducing effect of leukaemia virus.

Stimulation of virus producing cell populations by casein-antigens may well be the cause of the higher virus titre in casein-

treated than in saline treated infected mice (Siegel and Morton 1972)

Casein treatment stimulated the myeloid cell series in bone marrow since the number of peroxidase-positive cells growing in most cultures was increased after caseination. As this effect was not observed on bone-marrow cells obtained from thymus intact irradiated (T-cell reconstituted) mice, the marrow cell population in this group may be fundamentally different from that of the other groups. The involvement of the bone marrow population in amyloidogenesis is as yet virtually unexplored, the importance of bone marrow reactions for viral interference with amyloidogenesis therefore cannot be elucidated from the present results.

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PERSISTENT NEONATAL HYPOGLYCAEMIA

A Clinical and Histopathological Study of Three Cases Treated with Diazoxide and Subtotal Pancreatectomy

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Søvik, O Vidnes, J & Falkmer S. Persistent neonatal hypoglycaemia. A clinical and histopathological study of three cases treated with diazoxide and subtotal pancreatectomy Acta path. microbiol. scand. Sect. A, 83 155–166 1975.

Two boys and one girl developed persisting hypoglycaemia 12, 24 and 48 hours after birth. Although there was no known history of hereditary diabetes mellitus, the birth weight was high in two cases, and some additional traits of *foet pathia diabetica* could not be excluded clinically All had high serum insulin levels with frank hyperinsulinaemia in one case. Glucose tolerance tests also indicated hyperinsulinism. Diazoxide (8 to 27 mg/kg) elevated the blood glucose levels, but did not prevent severe hypoglycaemic episodes. The effects of subtotal pancreatectomy were only transient. The patients have now been kept on permanent diazoxide therapy for 2–4 years at dosages slightly lower than those used before operation. No islet-cell tumour was found at the subtotal pancreatectomy In all 3 cases, the pancreatic islets were markedly hyperplastic and of irregular shape with the occurrence of large B-cells with giant hyperchromatic nuclei and chromophobe "agranular" or sparsely granulated cells. The predominating kind of islet cells showed tinctorial features of A_1-cells, but—in the absence of available material set aside for ultrastructural analysis—it could not be settled whether this was a result of a proliferation of A_1-cells only or of "type IV cells" as well. Against the background of previously published reports, the present cases serve to illustrate that additional accuracy of diagnosis and classification of neonatal persistent hypoglycaemia requires quantitative information about the structural changes in the pancreatic islet cells, and that this can be obtained from conventional biopsy specimens.

Key words. Hypoglycaemia islands of Langerhans hyperinsulinism pancreatectomy; diazoxide.

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Most cases of neonatal hypoglycaemia are transient and easily controlled by frequent meals and intravenous glucose administration. In some instances, however the episodes of hypoglycaemia are frequent and severe, and continue after the neonatal period. Such cases frequently present great diagnosti problems (21) Furthermore, the therapeuti management of these cases is still unsatisfac tory and brain damage is difficult to avoid

The histopathological changes in the pan creatic islets in neonatal persistent hypo glycaemia of this kind are incompletel known (21) The number of structurally in vestigated cases is low and the results ar controversial, probably due to the fact tha

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modern differential granule staining procedures, including silver impregnation techniques (14 18) for islet parenchymal cells have rarely been applied. Moreover the pancreatic islets have only exceptionally been analysed by quantitative morphometric methods (21)

We think that a detailed structural analysis of the endocrine pancreas, using an adequate quantitative technique, is needed in cases of persistent neonatal hypoglycaemia in order to establish an unequivocal diagnosis and to improve the classification of the disease. The fact that a fair structural analysis is possible to perform also in cases where only conventional biopsy specimens are available, is illustrated by the present detailed report on 3 cases of severe and prolonged hypoglycaemia treated with a combination of subtotal pancreatectomy and diazoxide. In 2 of these cases, evidence has been obtained of a reduced *glucose production by the gluconeogenetic* pathway and all had a marked hyperplasia of the pancreatic islets. This paper is mainly concerned with clinical and histopathological findings and the response to treatment. Metabolic studies using ^{14}C-labelled alanine and glycerol in these cases will be described in a subsequent report (27)

METHODS

Plasma was obtained from heparinized blood by centrif gation as soon as possible after venipunc ure and kept t –20 C until analysed. Plasma imoreactive insulin (IRI) was determined by he method of *Hale & Randle* (15) (as described n detail in Technical Bulletin 68/6 The Radiochemical Centre, Amersham) Glucose in capillary blood was determined by the method of *Hultman* (19) The glucagon tolerance test was performed y following the blood glucose level after a rapid ntravenous (i) injection of glucagon (0.02 mg/ g) For the tolbutamide tolerance test, 20 mg per g was given at constant rate of i v infusion during 3 min. The i glucose tolerance test was performed by a constant nfusion of 0.5 g glucose per kg during 3 min The k-value for the glucose disappearance rate was calculated as described by *Amatuzio et al.* (1) The preparation of the pa ents for the tolerance tests was difficult since they did not tolerate an overnight fast The tests were started in the morning after varying periods of time without food, at least 3 hours.

The specimens obtained at the subtotal pancreatectomy included in all 3 cases not only *cauda pancreatis* but also *corpus* and parts of the *caput* The length of specimens was 4–6 cm and they were about 1.5 cm thick and 1–1.5 cm broad. The weight was about 3–5 g. They were fixed in conventional 10 per cent formalin. About 3 slices (cross-sections) from each of the fixed specimens were dehydrated, cleared, and embedded in paraffin. Sections, about 5 μ thick, were cut from the paraffin blocks, rehydrated and stained with van Gieson's stain and by 3 modern differential granule staining techniques for the parenchymal cells of endocrine pancreas, namely the chromehaematoxylin-ponceau-fuchsin procedure (20) for visualizing A (both A_1 and A_2) and B-cells, *Grimelius'* (14) silver nitrate method for detecting A_2 (and A)cells, and Davenport's silver impregnation technique as modified by *Hellerström & Hellman* (16) for identifying A_1-cells. The relative proportions between endocrine and exocrine pancreatic parenchyma were estimated by planimetry of selected areas of sections silver-impregnated according to *Grimelius* (14) (Fig 4) using a modified *Törnqvist* (23) procedure. Between 20 and 27 islets (and surrounding exocrine parenchyma) were measured in each case, and each planimetric measurement was repeated two times. The average difference between these 3 measurements aried from 0 8 to 3.7 per cent of the mean of each assessment, indicating the error of the method. The relative proportions of A_1 A_2 and B-cells were estimated by differential counting of about 1500 islet cells in each of the 3 *differential staining* procedures used No specimens for transmission electron microscopic investigations were taken.

Surgical liver biopsy specimens taken in all 3 patients in connection with the partial pancreatectomy measured about 1 × 1 × 0.5 cm and were fixed in formalin, After embedding in paraffin, cut sections were stained with van Gieson's stain. Best's carmine and the periodic-acid-Schiff (PAS) reagent were used to assess the *glycogen content of* the liver cells light microscopically

CASE REPORTS AND RESULTS

Case 1 B.S., a boy (birth weight 3 750 g length 50 cm) was born at term (*Jan. 29 1969*) after a normal pregnancy The mother was non-diabetic. Her glucose tolerance was not studied. Two cousins on his father side suffered from cerebral palsy and mental retardation, respectively B.S. was *cyanotic* at birth and did not cry the first 2 min. On the 3rd day of life he had convulsiv movements in the upper extremities, lasting for about

½ min., and at the age of 4 weeks a similar seizure was observed. No treatment was started, however and he was without symptoms up to his 7th week of life At this time more frequent seizures occurred, usually in the morning. Treatment was started with phenobarbital, without success, and at the age of 10 weeks he was admitted to the local paediatric ward. The most remarkable laboratory result was a blood glucose value of 16 mg per 100 ml. Treatment with i.v. glucose, Prednisone and growth hormone was unsuccessful. Plasma insulin determination suggested hyperinsulinism, and he was therefore at the age of 3 ½ months transferred to the Department of Paediatrics, Rikshospitalet, Oslo for further evaluation. Since the fasting IRI-levels were high, treatment with diazoxide was started at the age of 4 months. On a daily dose of 12 mg per kg, increasing blood glucose values and decreasing IRI-levels were observed. He was discharged from the hospital, but at the age of 6 months he was readmitted due to recurrent hypoglycaemic seizures. Blood glucose values below 25 mg per 100 ml were observed During the following months, the dose of diazoxide was gradually increased to 22 mg per kg However his hypoglycaemia remained difficult to control and, after a series of blood glucose values below 30 mg per 100 ml., he was subjected to a subtotal pancreatectomy (4/5) at the age of 11 months. However the postoperative blood glucose increase was transient only and treatment with diazoxide was resumed. At the age of 6 years he is still requiring the drug but his blood glucose level is usually well controlled by a dose of 8 mg per kg. A moderate hypertrichosis, present earlier has now disappeared He has a marked strabismus which needs operative correction. This boy has a rather easily controlled epilepsy and a moderately delayed speech development. His amino acid patterns in blood and urine have not been studied. No leucine sensitivity tests were made.

Case 2 L.M., a girl (birth weight 4,800 g, length 50 cm) was born at term (Oct. 12, 1970) after a normal pregnancy Her mother was non-diabetic and a glucose tolerance test was normal a sister of the patient had died 8 hrs after birth the mechanism of her death was unexplained. L.M. appeared somewhat plethoric otherwise there were no symptoms immediately after birth After a few hours however she had generalized convulsions with cyanosis and, the day after birth, she was transferred to the Department of Paediatrics Rikshospitalet, Oslo. At admission she had a blood glucose value of 10 mg per 100 ml. During the first days in the ward she had several hypoglycaemic seizures with blood glucose values below 30 mg per 100 ml. No leucine-sensitivity tests were made. She was treated with i.v. glucose and adrenocortical steroids without success and, after 2 weeks, diazoxide treatment was started. The dose of diazoxide was gradually increased to 24 mg per kg, but in spite of this her blood glucose was extremely difficult to control. Therefore at the age of 5 months a subtotal pancreatectomy (5/6) was performed. After the operation there was only a transient improvement of the condition and treatment with diazoxide was resumed At the age of 4 years she is fairly well controlled on diazoxide 4 mg per kg. However there is a marked tendency to low blood glucose values during infections and anorexia. Her amino acid patterns in blood and urine are both within the normal range. A marked hypertrichosis, present earlier has almost disappeared. She has a marked strabismus. The girl has an epilepsy which is difficult to control and EEG reveals signs of brain damage. She can just walk without support and speak single words.

Case 3 K.H., a boy (birth weight 4,210 g, length 51 cm) was born 3 weeks before expected time (Dec. 31 1971) after a normal pregnancy His mother was not diabetic, but it was not checked whether her glucose tolerance was normal. He had an Apgar score of 9 after one min. Subsequently he turned cyanotic, but improved on oxygen treatment. On the second day of life he had generalized convulsions and was transferred to the local paediatric ward where a blood glucose value of 27 mg per 100 ml was observed. Haemoglobin was 21.6 per 100 ml red blood cells 6.66 mill. per mm³ haematokrit 78 per cent. He was treated with i.v. glucose and 30 ml of blood was removed during venesectio During the following days he had frequent attacks of cyanosis and occasional apnoeic episodes. In spite of glucose infusion, blood glucose values below 20 mg per 100 ml were observed. After one week his general condition improved. However at 4 weeks of age he had a septicaemia with growth of *E. coli* from the blood. Treatment with antibiotics was successful and he again improved. Attempts to discontinue the glucose infusion, however were unsuccessful and treatment with cortisone was started. At the age of 8 weeks he was transferred to the Department of Paediatrics, Rikshospitalet, Oslo. Clinical examination at this time revealed a marked general retardation and bilateral atrophy of the optic nerve. At the age of 3 months, treatment with cortisone was discontinued and diazoxide was given. He received a dose of 27 mg per kg without satisfactory result and was therefore subjected to a subtotal pancreatectomy (5/6) when he was 3½ months old. After the operation there was only a transient blood glucose increase and treatment with diazoxide was resumed. Eventually his hypoglycaemia was controlled by a dose of 13 mg per kg. However hypoglycaemic episodes during fever still occurred. During the neonatal period a hyperglycinaemia was observed but it disappeared subsequently and his amino acid patterns in blood and urine are now normal. Any leucine-sensitivity tests have not been

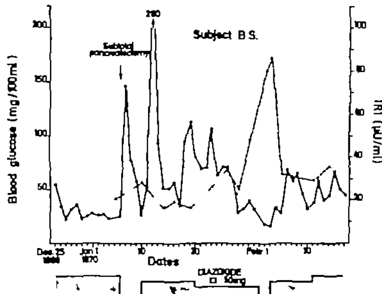


Fig 1 Blood glucose ●——● and plasma insulin (IRI) values ▲- - - -▲ in case 1 before and after subtotal pancreatectomy. The blood for glucose and insulin analysis was obtained in the morning after the patient had been without food for at least 3 hours. The dose of diazoxide is shown in the lower part of the figure.

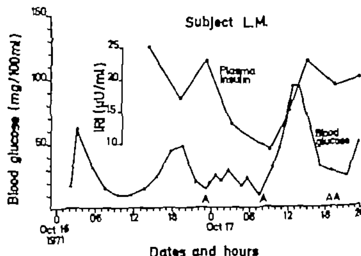


Fig 2 Blood glucose and plasma insulin (IRI) values in case 2. The data were obtained on her 5th and 6th day of life, when her blood glucose level was extremely difficult to control. A = attacks of hypoglycaemic seizures.

made. The brain of this boy is badly damaged, epilepsy is manifest and EEG is abnormal. No definite side effects of the drug have been observed.

Insulin, Blood Glucose and k-Values

In *case 1* studies of IRI before operation and treatment with diazoxide showed 127 µU per ml in the fasting state and 157, 126 and 129 µU per ml 1, 2 and 4 hours after a meal. On diazoxide treatment the fasting IRI-levels were markedly depressed (about 10 µU/ml) but in the pre-operative period, the blood glucose values being below 30 mg per 100 ml, fasting IRI values of about 20 µU per ml were observed (Fig 1). Postoperatively the IRI-levels varied between 16 and 34 µU per ml, even on diazoxide treatment, and during an attempt to discontinue the drug there was rise of IRI to 86 µU per ml. The fasting IRI was immediately brought down by resuming the medical treatment and later stabilised in the range of 5–15 µU per ml. The postoperative k-value in this case was 3.4 per cent per min.

Fig. 2 shows a series of IRI and blood glucose determinations in *case 2* in the early neonatal period before treatment with diazoxide was started. The IRI-level varied between 9 and 25 µU per ml, the blood glucose values ranging from 10 to 95 mg per 100 ml. Further pre-operative studies showed fasting IRI-levels reaching a maximum of 30 µU per ml at a blood glucose concentration of 112 mg per 100 ml. A postoperative tolbutamide tolerance test, when the patient was on diazoxide treatment,

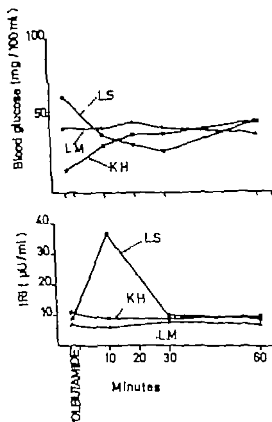


Fig 3 Blood glucose and plasma IRI response to tolbutamide. Case 2 (L.M.) and case 3 (K.H.) are compared with a hypoglycaemic patient (L.S.) (not belonging to the present series of three patients) who had not been pancreatectomized, but was on treatment with diazoxide. The tolbutamide tolerance test was performed as mentioned in "Methods"

showed a completely normal fasting IRI and lack of response to tolbutamide (Fig. 3) The postoperative k-value in this case was 3.8 per cent per min.

In *case 3* the first IRI-determination was performed at 2 weeks of age after a few days on cortisone treatment and showed 39 µU per ml at a blood glucose concentration of 50 mg per 100 ml. After 6 weeks on cortisone treatment, the following IRI-values were observed (corresponding blood glucose values in parentheses): 31 µU per ml (183 mg per 100 ml) 34 (112) 25 (79) 26 (58) 25 (41) 28 (38) The pre-operative k-value was 5.1 per cent per min. As in case 2, a tolbutamide tolerance test some months after operation during treatment with diazoxide revealed a normal fasting IRI-level and lack of response to tolbutamide (Fig 3)

Some pertinent data from all the 3 cases have been summarized in Table 1

Liver Biopsy and Glucagon Tolerance Tests

A routine histopathological examination revealed nothing abnormal in any of the liver biopsy specimens from the 3 patients. There seemed to be normal amounts of glycogen in the cytoplasm of the hepatocytes.

These histopathological observations were in conformity with the fact that the glucagon tolerance tests gave a glycaemic response well within the normal range in all 3 cases.

Islets of Langerhans

The structural changes observed in pancreas were essentially the same in all 3 cases. Already at a cursory examination a marked hyperplasia and hypertrophy of the islets of Langerhans could be established (Fig. 4) The acinar parenchyma was normal and the ducts showed no abnormalities.

TABLE 1 *Clinical Findings and Laboratory Data in 3 Infants with Persistent Neonatal Hypoglycaemia*

Case	Birth weight (g)	Age at onset of symptoms	Glucose disappearance rate (per cent/min.)	Fasting IRI before treatment (µU/ml)	Follow-up examination
1 (B.S.)	3 750	2 days	3 40	127	Slight retardation. Epilepsy Strabismus.
2. (L.M.)	4,800	12 hours	3 75	10–25	Moderate/marked retardation. Epilepsy Strabismus.
3. (K.H.)	4,210	1 day	5.15	25–39	Marked retardation. Epilepsy Optic nerv. atrophy

The glucose disappearance rate (k-value) was calculated as described under "Methods"



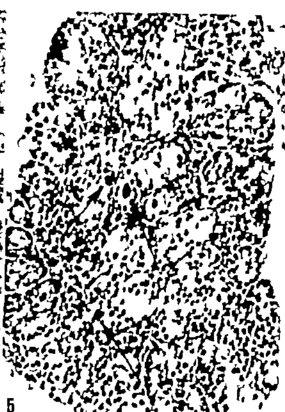


Fig 4 Case 1 Low-power photomicrograph of a section of the pancreas, stained by *Grimelius'* (14) silver nitrate procedure in order to visualize the A_2-cells. As these are mainly located in the periphery and occur in all islets, the argyrophil A_2-cells (dark) at the same time show the size, shape and number of *islets in the section. It is obvious that there is a marked hyperplasia and hypertrophy of the endocrine* pancreas. By planimetry it was found that in this area the islets occupied almost ½ of the total area of pancreatic parenchyma (normal value for children of this age 2–3 per cent) (21) Similar observations were made in cases 2 and 3 × 105

Fig 5 Case 2. Medium-power photomicrograph of a small lobule of the pancreatic parenchyma, showing a hyperplastic, rather ill-defined islet, occupying most of the central parts of the lobule. There is a considerable variation in size of the islet cells and some are hypertrophic with large hyperchromatic nuclei (arrow) These cells are either B-cells or chromophobe "agranular" or sparsely granul ted cells. (Most of the islet parenchymal cells were however A_1 or A_2-cells *cf* Fig 4) van Gieson's stain. × 270.

There were no inflammatory changes and no islet cell tumours were discovered.

Several hyperplastic islets contained parenchymal cells the size of which varied markedly Some hypertrophic cells showed giant hyperchromatic nuclei (Fig 5) These lls were either B-cells or chromophobe granular" or sparsely granulated non-argyrophil cells.

The results of the quantitati e light microscopic analysis of the pancreatic glands n our 3 cases are given in Table 2. It is lear that a marked relati increase of the endocrine parenchyma had occurred in all the 3 cases, being particularly obvious in case 1 where also a frank hyperinsulinism was established.

In chrome-haematoxylin-ponceau-fuchsin stained sections, about 60 per cent of the islet cells showed staining characteristics of A and A_2-cells, about 40 per cent were B-cells, and 2 per cent just took a faint background stain and were classified as chromophobe "agranular or sparsely granulated cells. In sections stained according to *Grimeli* (14) this interrelationship between A and B-cells was confirmed. Between 52 and 57 per cent of the islet parenchymal cells were argyrophil (Fig. 4) Thus a marked proliferation of A_2-cells had obviously occurred in all 3 cases. Against this background it was surprising to find in sections silver-impregnated according to *Hellerström & Hellman* (18) *that the relati number of cells staining like*

TABLE 2. *Quantitative Assessment of the Islets of Langerhans in 3 Infants with Persistent Neonatal Hypoglycaemia*

Specimen of pancreas	Islet tissue in per cent of total pancreatic parenchyma	Various kinds of islet cells in per cent of total number of parenchymal cells in endocrine pancreas			
		B-cells§	A_2-cells†	A_1-cells‡	Large clear chromophobe cells§
Case No. 1 (B.S.) (11 months old)	18 ± 1.7□	43	52	15	3
Case No. 2 (L.M.) (5 months old)	10 ± 3.8□	40	57	12	2
Case No. 3 (K.H.) (3/4 months old)	11 ± 3.9□	43	57	15	2
Mean of 10 normal infants, 4–8 months old	2 (1.3–3.0)δ	57 (50–60)δ	18 (10–20)δ	24 (20–35)δ	0

As found in sections stained by *Grimelius'* (14) procedure (Fig. 4). *Single islet cells lying disseminated* in duct epithelium or in the acinar parenchyma are not included.

§ As found in sections stained with chrome-haematoxylin-ponceau-fuchsin (20).

† *Grimelius'* (14) procedure.

‡ *Hellerström & Hellman's* (18) method.

□ Standard error of the mean. Between 20 and 27 islets were measured in each case.

δ Range.

The relative proportion of islet parenchyma was determined by planimetry and the percentage of various kinds of islet *parenchymal* cells was obtained by *differential cell countings on adjacent sections stained by* the chrome-haematoxylin-ponceau-fuchsin method (B-cells *vs* non-B-cells), by *Grimelius'* (14) silver nitrate procedure (A_2-cells *vs.* non-A_2-cells) and by *Hellerström & Hellman's* (18) modification of the Davenport silver impregnation procedure (A_1-cells *vs.* non-A_1-cells). The results are compared with some data from the literature (21) originating from allegedly normal infants of similar age.

A-cells was 12–15 per cent, indicating that either so-called "type IV cells" (5, 6, 21) or some B-cells (26) and/or some A_2-cells (14) had been included in this category. The topographic localization of the argyrophil A- and A_2-cells in the islets was essentially the same.

Effect of Treatment

The effect of diazoxide treatment of the 3 cases is summarized in Table 3. On a dose varying between 22 and 27 mg per kg, definite blood glucose increase was observed in all 3 cases. However the increase was either moderate as in case 1 or there were still episodes of severe hypoglycaemic seizures, as in cases 2 and 3. The only side effect observed was *hypertrichosis lanuginosa in case 1* (mild) and case 2 (severe). In Table 4 the results of subtotal pancreatectomy are summarized. In all 3 cases, the postoperative blood glucose increase was transient. The daily blood glucose values in the 3 patients before and after operation are presented in Figs. 1, 6 and 7. The postoperative

TABLE 3. *Results of Diazoxide Therapy in 3 Infants with Persistent Neonatal Hypoglycaemia*

Case	Age at onset of treatment	Duration (years)	Dose (mg/kg)		Blood glucose increase	Side effects
			Before operation	After operation		
1 (B.S.)	4 months	5	8→22	22→8	Moderate	Hypertrichosis, slight
2 (L.M.)	2 weeks	4	16→24	14→4	Slight	Hypertrichosis, marked
3 (K.H.)	3 months	3	9→27	21→13	Slight	None

TABLE 4 *Results of Subtotal Pancreatectomy in 3 Infants with Persistent Neonatal Hypoglycaemia*

Case	Pancreatectomy*	Age at operation	Results	
			Operation	Operation + diazoxide
1 (B.S.)	4/5	11 months	Transient blood glucose increase	Normoglycaemia
2. (L.M.)	5/6	5 months	Transient blood glucose increase	Normoglycaemia
3 (K.H.)	5/6	3½ months	Transient blood glucose increase	Normoglycaemia

* The relative proportion of pancreatic tissue removed is indicated by the fraction given in this column.

hyperglycaemia was of short duration and was followed by normoglycaemia and even hypoglycaemia within a few days. Thereupon a second period of hyperglycaemia occurred, followed by gradually decreasing glucose levels. All 3 cases required diazoxide after operation the doses were almost as high as before pancreatectomy but the sensitivity to the drug was apparently increased. In case 1 the diazoxide treatment was resumed as early as on the fourth postoperative day. During an attempt to discontinue the drug treatment in this case, the boy developed hypoglycaemia and hyperinsulinism and the treatment was immediately resumed (Fig. 1)

DISCUSSION

The hypoglycaemia of the 3 cases presented was characterized by an early onset and persistence beyond the neonatal period. Cases 2 and 3 had high birth weights. Also in other clinical respects they resembled *foetopathia diabetica* without being actual offsprings of diabetic mothers. Plasma insulin determinations performed before treatment with diazoxide and subtotal pancreatectomy revealed rather high levels in all 3 cases as compared

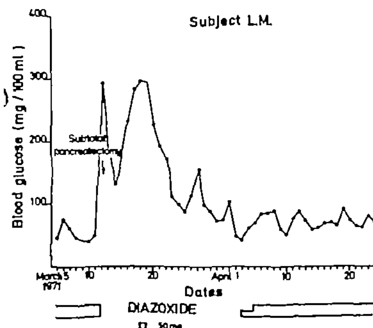


Fig. 6 Blood glucose values in case 2 before and after subtotal pancreatectomy. The blood glucose values indicated are the means of 2 or more daily determinations. The daily dose of diazoxide is shown in the lower part of the Figure

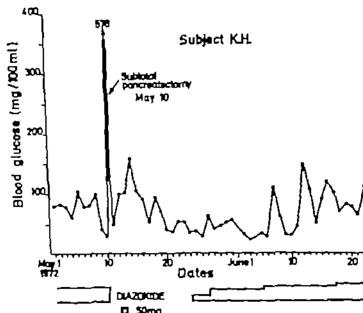


Fig. 7. Blood glucose values in case 3 before and after subtotal pancreatectomy. The blood for glucose analysis was obtained in the morning after the patient had been without food for at least 3 hours. The daily dose of diazoxide is shown in the lower part of the Figure.

with the glucose values. However except in case 1 there was no frank hyperinsulinism.

The structural analysis of the endocrine pancreas in all 3 cases was invalidated by the facts that the specimens obtained at subtotal pancreatectomy were only fixed in formalin, giving rather bad preservation of the islet parenchymal cells, and that no material was set apart for transmission electron microscopy. Recent reports (21, 22) on other cases of neonatal hypoglycaemia have shown that a fourth type of granulated parenchymal islet cells, the so-called type IV cells (5), occurring also in normal islets of infants and children (6), can be found in fairly high relative proportions in this disease. Light-microscopically these "type IV cells" cannot be differentiated from A_1-cells (21). This is only possible by ultrastructural investigations. Consequently it was impossible to prove that "type IV cells" were present in the islets of our 3 cases.

It is not known whether the "type IV cells" are argyrophil also by *Grimelius'* (14) procedure, but preliminary studies from our laboratory and by others (*Creutzfeldt*, personal communication) indicate that this may be the case. Valuable contributions to pancreatic islet cytology in general have been obtained from light-microscopical and ultrastructural studies of cases of neonatal hypoglycaemia where it has been shown that, in these states, about 25 per cent of the islet cells are "type IV cells" and that the light-microscopically defined A_1-cells correspond ultrastructurally to D-cells as well as to "type IV cells" (21). As the pancreatic islets of our 3 cases in all other respects showed pictures almost identical to those described in preceding reports on persistent neonatal hypoglycaemia, it seems reasonable to assume that they also showed a marked increase in the relative frequency of "type IV cells". If our preliminary observations that these "type IV cells" are argyrophil also by *Grimelius* (14) technique are correct, this might be an explanation of the marked relative increase in the number of A_2-cells and also of the surprising observations that the relative number of argyrophil cells by the *Hellerström & Hellman* (18) procedure was proportionately too high in all 3 cases. In man, the *Grimelius'* (14) method is said to stain A_2-cells almost selectively. The relative frequency of the A_1, A_2- and B-cells in normal infants of 4–8 months of age is given in Table 2. "Type IV cells" and chromophobe "agranular" or

TABLE 4 *Results of Subtotal Pancreatectomy in 3 Infants with Persistent Neonatal Hypoglycaemia*

Case	Pancreatectomy*	Age at operation	Results	
			Operation	Operation + diazoxide
1 (B.S.)	4/5	11 months	Transient blood glucose increase	Normoglycaemia
2 (L.M.)	5/6	5 months	Transient blood glucose increase	Normoglycaemia
3. (K.H.)	5/6	3½ months	Transient blood glucose increase	Normoglycaemia

* The relative proportion of pancreatic tissue removed is indicated by the fraction given in this column

hyperglycaemia was of short duration and was followed by normoglycaemia and even hypoglycaemia within a few days. Thereupon a second period of hyperglycaemia occurred, followed by gradually decreasing glucose levels. All 3 cases required diazoxide after operation; the doses were almost as high as before pancreatectomy, but the sensitivity to the drug was apparently increased. In case ... the diazoxide treatment was resumed as early as on the fourth postoperative day. During an attempt to discontinue the drug treatment in this case, the boy developed hypoglycaemia and hyperinsulinism and the treatment was immediately resumed (Fig 1).

DISCUSSION

The hypoglycaemia of the 3 cases presented was characterized by an early onset and persistence beyond the neonatal period. Cases 2 and 3 had high birth weights. Also in other clinical respects they resembled *foetopathia diabetica* without being actual offsprings of diabetic mothers. Plasma insulin determinations performed before treatment with diazoxide and subtotal pancreatectomy revealed rather high levels in all 3 cases as compared

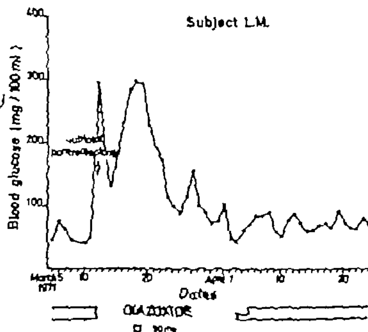


Fig 6 Blood glucose values in case 2 before and after subtotal pancreatectomy. The blood glucose values indicated are the means of 2 or more daily determinations. The daily dose of diazoxide is shown in the lower part of the Figure

secretion in cases 2 and 3 did not provide a sole and satisfactory explanation of their hypoglycaemia, studies of the glucose production by gluconeogenesis were undertaken. This work will be presented in a subsequent paper (27).

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The subtotal pancreatectomies were performed by Dr *O. Knutrud* Dept. of Paediatric Surgery Rikshospitalet, University of Oslo.

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ENZYMATIC HETEROGENEITY OF GRANULES IN HUMAN LEUCOCYTES

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Lundgren, E., Roos, G. & Tärnvik, A. Enzymatic heterogeneity of granules in human leucocytes. Acta path. microbiol. scand. Sect. A, 83: 167–175, 1975.

Homogenates of highly purified polymorphonuclear leucocytes and of a mixture of mononuclear leucocytes and platelets from human blood were separated by differential and isopyknic centrifugation. A heterogeneity in granules containing digesting enzymes was found in both cell preparations. Enzymes typical of lysosomes were found in the two cell preparations in a similar density range. Granules of low density were indicated in polymorphonuclear leucocytes by alkaline phosphatase. In both cell preparations a third granule, of lower density, seemed to exist enriched in amino acid naphthylamidase, acid hydrolases and in polymorphonuclear leucocytes also alkaline phosphatase and lysozyme. A remarkable difference between the two cell preparations was the occurrence of amino acid napthylamidase in denser granules of polymorphonuclear leucocytes only, although the nature of these granules could not be determined.

Key words: Leucocytes; lysosomes; amino acid naphthylamidase; electron microscopy.

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Polymorphonuclear leucocytes may digest a microorganism into non-immunogenic products, whereas the same microorganism is digested by mononuclear phagocytes into pieces which are still immunogenic (*Cohn* 1962). The basis for this difference is unknown.

Many digesting, mainly lysosomal, enzymes have been demonstrated in polymorphonuclear leucocytes (*cf. Weissman* 1967) and in mononuclear phagocytes (*cf. Pearsall & Russell* 1970). Granules containing digesting enzymes are heterogenous with respect to enzyme content, size or density. In polymorphonuclear leucocytes from rabbit peritoneal exudates four particulate components have been characterized (*Baggiolini et al.* 1969, 1970, *Farquhar et al.* 1972). A granular heterogeneity has also been found in human blood polymorphonuclear leucocytes (*Baggiolini* 1972, *Welsh & Spitznagel* 1971), but there are differences between polymorphonuclear leucocytes from human peripheral blood and rabbit peritoneal exudates (*Spitznagel* 1972, *West & Kimball* 1972). There is some evidence of a heterogeneity in lysosomal enzyme distribution, not only in polymorphonuclear but also in mononuclear leucocytes (*Bowers & deDuve* 1967, *Nichols et al.* 1971).

There is virtually no data as regards the subcellular localization of digesting enzymes by which blood cells can be compared. The aim of the present investigation was to study the subcellular localization of digesting enzymes in highly purified polymorphonuclear

leucocytes and in mononuclear leucocytes from human blood. Conventional markers of granule populations (*Baggiolini et al.* 1969) were used as well as amino acid naphthylamidase, a peptidase with broad substrate specificity. There was a marked difference between the polymorphonuclear and mononuclear cell preparations with respect to the intracellular distribution of alkaline phosphatase and amino acid naphthylamidase.

MATERIAL AND METHODS

Each sample of 400 ml venous blood was obtained from a healthy blood donor and collected into a sterile bottle containing 6,000 IU of heparin in 4 ml saline. Cells were fractionated according to a modification of the technique of *Pertoft et al.* (1968) described in detail previously (*Tõrncik* 1970). After sedimentation of the blood, the red cell poor supernatant was centrifuged on colloidal silicapolyvinyl-pyrrolidone.

Two preparations were obtained, the PMN and MN preparations. The former contained 0–7 lymphocytes, 10–40 red cells and a *few* platelets per 100 polymorphonuclear leucocytes. No monocyte was found among 400 leucocytes.

The MN preparation contained lymphocytes, monocytes and platelets in about the same relative proportion as in whole blood, about 3 PMN cells and fewer than 1 red cell per 100 leucocytes.

The cell preparations were washed twice in 14 per cent polyethyleneglycol supplemented with 2 per cent foetal calf serum and once in 0.25 M sucrose, pH 7.0.

Washing and fractionation of all preparations were performed at 0–4° C. After the last washing, one volume of cells was suspended in 20 volumes of 0.25 M sucrose. Homogenization was performed with the tight fitting pestle of a Dounce homogenizer and the degree of homogenization was followed in phase contrast microscope. Three to six strokes were sufficient to obtain less than 5 per cent intact cells.

Differential centrifugation was performed according to established procedures (*Cohn & Hirsch* 1960) except that the sucrose concentration was 0.25 M. The homogenate was centrifuged in an ordinary laboratory centrifuge at 400 g for 10 min. The resulting pellet was washed once, recentrifuged and then suspended in sucrose. It corresponded to the nuclear fraction of *Cohn & Hirsch*. The combined 400 g supernatants were centrifuged at 8,200 g for 15 min and the resulting pellet, i.e., the granular fraction, was suspended in sucrose. Hence, the 8,200 g supernatant is equivalent to the postgranular fraction. The 400 g pellet, the 8,200 g pellet and the 8,200 g supernatant were stored at –20° C until the enzyme analyses were performed.

The 400 g supernatant was subjected to density equilibrium gradient centrifugation in a swing-out rotor (SW 56, Beckman) at 120,000 g for 2.5 hours. Two gradients were used (*Lundgren* 1971). One was made up of 25–55 per cent sucrose, the other of 0.5 M sucrose in 0–15.3 per cent glycogen. Ten subfractions were obtained by aspiration from the top of the gradient. In order to release latent enzyme activity in leucocyte granules, all fractions were subjected to ten cycles of freezing and thawing (–20° C to 4° C) before measuring the enzyme activities. By this procedure the activities of three enzymes, acid and alkaline phosphatase and amino acid naphthylamidase deviated less than ± 10 per cent from the activities released by the detergent Triton X 100. The results of the gradient experiments were represented graphically according to *Sloat & Allen* (1969). The recoveries of the measured enzyme activities were in the range of 90–105 per cent as related to the sample applied on the gradient.

Acid phosphatase was measured with β-glycerophosphate (*Lundgren* 1971) or p-nitrophenyl phosphat (*Michell et al.* 1970) as substrates in 0.05 M acetate buffer pH 5.0. β-glucuronidase was assayed according to *Brittinger et al.* (1968) with phenolphtalein-β-glucuronide as substrate in 0.05 M acetate buffer pH 4.6. Lysozyme was measured according to *Cohn & Hirsch* (1960) by recording the decrease in optical density at 450 nm of dried *M. lysodeikticus* suspended in 0.1 M phosphate buffer pH 6.5 in the presence of the sample. Alkaline phosphatase was assayed with p-nitrophenyl phosphate as substrate (*Michell et al.* 1970) in 0.05 M glycine-NaOH buffer pH 10.0 with 10 mM $MgCl_2$. Amino acid naphthylamidase was assayed according to *Lundgren* (1971) with L-leucyl-β-naphthylamide as substrate in 0.05 M phosphate buffer pH 7.0 and protein according to *Lowry et al.* (1951).

Acid p-nitrophenylphosphatase and alkaline phosphatase were expressed as μmol p-nitrophenol/mg protein/hour, acid β-glycerophosphatase as mmol P_i/mg protein/hour, β-glucuronidase activity as mmol phenolphtalein/mg protein/hour, amino acid naphthylamidase activity as μM β-naphthylamine/mg protein/hour and lysozyme as units/min. One unit was defined as the amount of enzyme causing a decrease in optical density of 1.0.

For the electron microscopy studies, subfractions from the gradients were diluted with 0.25 M sucrose and centrifuged at 50,000 g for 30 min. The pellets were fixed, embedded and stained according to *Nakamura et al.* (1972). After fixation thin films were scraped from the tubes and the small pieces obtained were dehydrated and em-

bedded in Epon. The pieces were sectioned at different levels as some of them were not homogeneous.

RESULTS

Differential Centrifugation

The homogenate of the MN preparation was subjected to differential centrifugation and separated into three fractions (the 400 g pellet, the 8,200 g pellet and the 8,200 g supernatant, Table 1) The bulk of acid β-glycero-phosphatase and β-glucuronidase *i.e.*, marker enzymes for lysosomes was recovered in the 8,200 g pellet, whereas alkaline phosphatase was more evenly distributed and amino acid naphthylamidase appeared predominantly in the 8,200 g supernatant. The recoveries of the enzymes were about 40 to 60 per cent in the respective, enriched fraction.

Differential centrifugation of the homogenate of the PMN preparation gave similar results. Lysozyme was enriched in the 8,200 g pellet (Table 2)

TABLE 1 *Subcellular Distribution of Protein Lysosomal Enzymes Alkaline Phosphatase and Amino Acid Naphthylamidase in the MN Preparation*

	400 g pellet	8,200 g pellet	8,200 g supernatant	Recovery	Specific activity of the original cell preparation
Protein (n = 3)	22.6 ± 4.1	33.3 ± 11.2	43.9 ± 7.0	100.0 ± 8.6	
Acid phosphatase (n = 3)	23.4 ± 5.0	42.0 ± 14.1	34.6 ± 9.2	80.1 ± 9.8	0.32 ± 0.03
β-glucuronidase (n = 3)	18.7 ± 4.2	55.1 ± 8.9	26.2 ± 7.7	120.2 ± 12.6	0.07 ± 0.0
Alkaline phosphatase (n = 3)	18.6 ± 1.4	31.3 ± 8.7	50.1 ± 8.2	105.6 ± 17.0	0.10 ± 0.02
Amino acid naphthylamidase (n = 5)	14.4 ± 3.1	24.9 ± 8.9	60.3 ± 9.7	89.6 ± 7.8	0.56 ± 0.07

The results are given as percentage of recovered activity expressed as mean ± SEM.

TABLE 2. *Subcellular Distribution of Protein Lysosomal Enzymes and Amino Acid Naphthylamidase in the PMN Preparation*

	400 g pellet	8,200 g pellet	8,200 g supernatant	Recovery	Specific activity of the original cell preparation
Protein (n = 5)	26.2 ± 3.0	34.5 ± 4.9	39.3 ± 5.2	88.0 ± 3.6	
Acid phosphatase (n = 3)	18.8 ± 5.6	55.4 ± 6.5	23.8 ± 1.5	83.7 ± 5.2	0.59 ± 0.08
β-glucuronidase (n = 6)	27.9 ± 5.2	60.7 ± 5.9	11.5 ± 1.6	91.4 ± 3.6	0.12 ± 0.01
Lysozyme (n = 3)	25.4 ± 10.5	61.0 ± 11.4	13.7 ± 1.2	91.8 ± 15.7	0.16 ± 0.02
Alkaline phosphatase (n = 6)	15.2 ± 4.5	29.0 ± 5.5	55.8 ± 5.2	99.7 ± 8.4	0.52 ± 0.13
Amino acid naphthylamidase (n = 5)	9.3 ± 3.8	34.[illegible] ± 8.7	56.3 ± 7.9	91.5 ± 5.4	0.72 ± 0.04

The results are given as percentage of recovered activity expressed as mean ± SEM

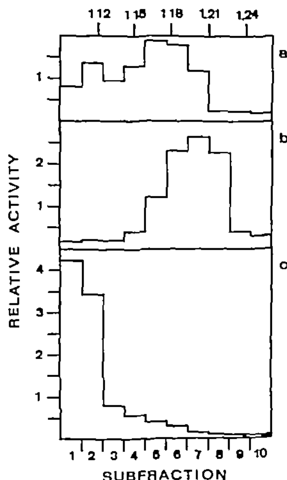


Fig 1 Sucrose-water density gradient subfractionation of the 400 g supernatant of the MN preparation. The relati e activity is calculated as the enzyme activity present in each fraction divided, by the calculated mean enzyme activity per fraction. a) acid β-glycerophosphatase b) β-glucuronidase) amino acid naphthylamidase.

Centrifugation in Sucrose Water Gradient

When the 400 g supernatant of the MN preparation was centrifuged in the sucrose-water gradient, acid β-glycerophosphatase and β-glucuronidase were recovered as a broad peak in subfractions 5–8 These enzymes were well separated from amino acid naphthylamidase which appeared in subfraction 1–2 (Fig. 1)

In the PMN preparation the distribution of acid β-glycerophosphatase and amino acid naphthylamidase was similar to that in the MN preparation (Fig 2) β glucuronidase

Fig 2 Sucrose-water density gradient subfractionation of the 400 g supernatant of the PMN leucocyte preparation. The relative activity is calculated as the enzyme activity present in each fraction, divided by the calculated mean enzyme activity per fraction a) acid β-glycerophosphatase b) acid p-nitrophenyl phosphatase c) β-glucuronidase d) lysozyme, e) alkaline phosphatase f) amino acid naphthylamidase

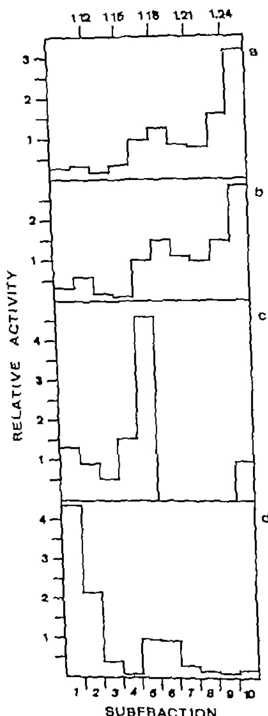


Fig. 3 Glycogen-sucrose density gradient subfractionation of the 400 g supernatant of the MN leucocyte preparation. The relative activity is calculated as the enzyme activity present in each fraction, divided by the calculated mean enzyme activity per fraction. a) acid β-glycerophosphatase, b) β-glucuronidase c) alkaline phosphatase d) amino acid naphthylamidase

lysozyme and acid p-nitrophenyl phosphatase were found in the same broad peak as acid β-glycerophosphatase Alkaline phosphatase appeared between the peaks of amino acid naphthylamidase and the acid hydrolases.

For morphological examination, subfractions 2–4 and 5–8 from the MN and PMN preparations, respectively were pooled, omitting subfraction 1 which contained soluble components only Subfractions 5–8 of the MN preparation contained homogenous lysosomal granules, whereas granules of the corresponding subfractions of the PMN preparation were heterogenous in size, shape and matrix structure.

In subfractions 2–4 of the MN preperation there were no granules, only membrane fragments and small vesicular material (Fig 5 a) whereas the same subfractions of the PMN preparation also contained granules of varying size and electron density at varying levels of the pellets (Fig. 5 b and c)

Centrifugation in Glycogen-Sucrose Gradient

After centrifugation of the 400 g supernatant of the MN preparation in the glycogen-sucrose gradient, the bulk of acid β-glycerophosphatase and β-glucuronidase was found at subfractions 9–10 but these enzymes also had a minor peak in subfractions 5–8 (Fig. 3) Alkaline phosphatase had a dual distribution. The main activity was recovered in the 5th subfraction, but activity was also concentrated in subfractions 1–2. Amino acid naphthylamidase was mainly concentrated in subfractions 1 2 although a minor peak also was seen in the 5–6th subfractions.

In the PMN preparation, acid phosphatase, β-glucuronidase lysozyme and alkaline phosphatase were recovered in subfractions 9–10 but also in this preparation minor peaks of activity were found in subfractions 5–6 alkaline phosphatase however in subfractions

4–6 (Fig. 4) Amino acid naphthylamidase appeared in subfractions 1–2 and 5–6 also in the PMN preparations. But in contrast to the MN preparation, there was also a peak at the bottom of the gradient (subfractions 9–10) This indicates that amino acid naphthylamidase activity was localized in granules enriched in either alkaline phosphatase or lysosomal enzymes.

It was not possible to examine morphologically the 9–10th subfractions of the glycogen gradient because of heavy contamination of large glycogen particles from the gradient. In subfractions 5–6 of the MN preparation membrane fragments were found, but also granules with the size and appearance of lysosomes. However the matrix was not homogenous, rather irregularly granular (Fig. 5 d) Also in the subfractions 5–6 of the PMN preparations there were cell membranes and granules of varying appearance. Membrane fragments were numerous, as well as small particles with the appearance of vesicules or empty granules (Fig. 5 e)

In conclusion, three granular fractions were obtained from human leucocytes One fraction, containing lysosome-like granules, was recovered in the density region of 1 16–1.22 in the PMN as well as in the MN cell preparation. Another fraction with a granular density of about 1 13 was enriched in alkaline phosphatase and was obtained only from PMN leucocytes. In the middle of the glycogen-sucrose gradient, both cell preparations exhibited a third type of granule fraction enriched in amino acid naphthylamidase but also containing lysosomal enzymes.

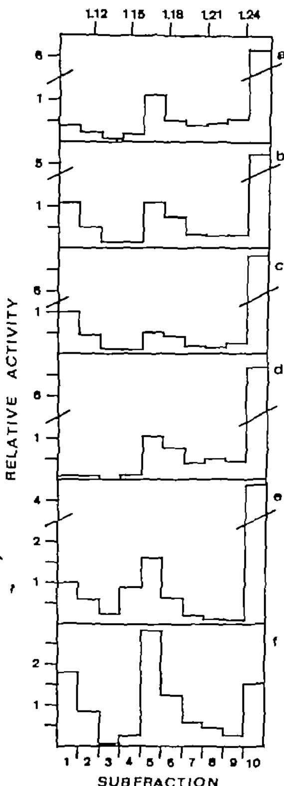


Fig 4 Glycogen-sucrose density gradient subfractionation of the 400 g supernatant of the PMN leucocyte preparation. The relative activity is calculated as the enzyme activity present in each fraction, divided by the calculated mean enzyme activity per fraction. a) acid β-glycerophosphatase b) acid p-nitrophenyl phosphatase c) β-glucuronidase d) lysozyme e) alkaline phosphatase f) amino acid naphthylamidase

DISCUSSION

PMN leucocytes of rabbit peritoneum have been extensively studied as they are easily



Fig 5 Electron micrographs from pooled subfractions obtained after gradient centrifugations.
a) MN preparation, sucrose-water gradient, subfractions 2–4 × 55,000
b) PMN preparation, sucrose-water gradient, subfractions 2–4 × 46,000
c) PMN preparation, sucrose-water gradient, subfractions 2–4 × 58,000
d) MN preparation, glycogen-sucrose gradient, subfractions 5–6 × 64,000
e) PMN preparation, glycogen-sucrose gradient, subfractions 5–6, × 58,000

available in large amounts. Their cytoplasmic granules are well characterized (*Baggiolini et al.* 1969 1970 *Farquhar et al* 1972, *Bretz & Baggiolini* 1973) Human PMN leucocytes are not as easily isolated and seem to contain a more heterogenous granule population. Therefore it has not been possible to achieve a close correlation between biochemical data and morphological structures (*Baggiolini* 1972, *Spitznagel* 1972) However there is evidence that human PMN leucocytes have lysosomes containing lysozyme and acid hydrolases, and also granules of a lower density enriched in alkaline phosphatase (*Olsson*

1969 *West & Kimball* 1971 *Spitznagel* 1972) These data are confirmed in the present study

Baggiolini et al. (1970) and *Spitznagel* (1972) localized acid p-nitrophenyl phosphatase in granules containing alkaline phosphatase, whereas the present results in agreement with those of *West & Kimball* (1971) suggest that this enzyme is enriched at a density similar to that of acid hydrolases. This discrepancy may be due to the different techniques used for cell separation. The present cell preparation was highly purified no monocyte was found among 400 PMN leucocytes.

The present glycogen-sucrose gradient was found to be suitable to extend the resolution in the low density region. Thereby subfractions were found, enriched in acid hydrolases, alkaline phosphatase and amino acid naphthylamidase in MN as well as in the PMN cell preparation. Some of the fragments or granules seen in these subfractions may be phagolysosomes and disintegrated lysosomes. Another possibility is that a microsomal organelle has this density. Acid phosphatase and amino acid naphthylamidase have been demonstrated in cultured HeLa cells in a microsomal fraction (*Lundgren* 1971)

A striking difference between the MN and PMN cell preparations is the presence of a dense granule containing amino acid naphthylamidase in the latter only. A localization of this enzyme in some kind of lysosomal granule or in the granules enriched in alkaline phosphatase is probable but cannot be determined from the present results.

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SUBCELLULAR DISTRIBUTION OF AMINO ACID NAPHTHYLAMIDASE ISOZYMES IN HUMAN LEUCOCYTES

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Lundgren, E., Roos, G & Tärnvik, A. Subcellular distribution of amino acid naphthylamidase isozymes in human leucocytes. Acta path. microbiol. scand. Sect. A, 83 176–182, 1975.

Three isozymes (A, B and C) of amino acid naphthylamidase were found in human leucocytes. Isozyme B and C had close biochemical similarities and differed from isozyme A. Isozyme A was localized in the cytosol. Results obtained by combining electrophoresis with gradient centrifugation suggested that isozyme B originated in granules of polymorphonuclear leucocytes enriched in alkaline phosphatase. Isozyme C was found in granules of low density of polymorphonuclear as well as mononuclear leucocytes.

Key words Leucocytes amino acid naphthylamidase isoenzymes.

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Amino acid naphthylamidase is localized in different subcellular compartments of mammalian cells (*Mahadevan & Tappel* 1967 *Brecher & Suszkiw* 1969 *Lundgren* 1971 b) Soluble lysosomal and microsomal activities appear in different molecular forms, *i.e.* isozymes.

Few data on the localization of amino acid naphthylamidase in leucocytes are available. In polymorphonuclear leucocytes, the predominant activity has been found in a fraction with a density lower than that of lysosomes (*Davies et al* 1970 *Folds et al.* 1972) The activity of a polymorphonuclear leucocyte preparation (PMN preparation) has been enriched in three subcellular fractions (*Lundgren et al* 1974) One fraction is the cytosol and the others seem to be granules of low and high density In a preparation containing mononuclear leucocytes and platelets (MN preparation) a similar distribution has been found except for the lack of activity corresponding to the denser granules.

In the present study the amino acid naphthylamidase activities of subcellular fractions of human PMN and MN preparations were distinguished as different isozymes, localized in different granule types.

MATERIAL AND METHODS

Separation and homogenization of PMN and MN preparations of human blood have been described in a preceding paper (*Lundgren et al* 1974)

Cell homogenates were centrifuged at 400 g for 10 minutes to get rid of nuclei, cell debris and unbroken cells. The supernatant was subjected to density equilibrium centrifugation in sucrose-water and glycogen-sucrose gradients as previously described (*Lundgren et al.* 1974)

Amino acid naphthylamidase activity was ex

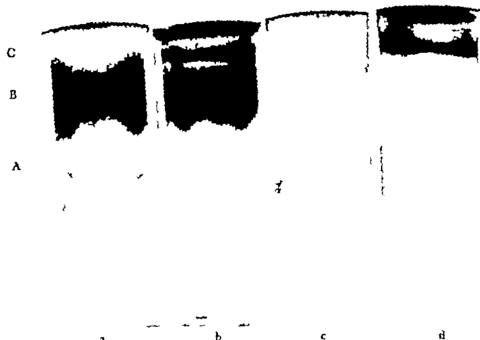


Fig. 1. Zymogram of amino acid naphthylamidase on polyacrylamid gels. Applied material was obtained from PMN (a and b) and MN (c and d) preparations. Extracts were obtained by freezing and thawing of the homogenates (a and c) and by Triton X 100 treatment of the still insoluble activity (b and d). One of the three isozymes found, isozyme B, consisted of two components which could be revealed by longer running distances.

tracted from cell homogenates by ten cycles of freezing at −20 C and thawing at +4 C followed by centrifugation at 105,000 g for 60 minutes. Extraction of more than 90 per cent of enzyme activity remaining in the pellet was done by treatment with 1 per cent Triton X 100 in 0.25 M sucrose for one hour at 4 C followed by centrifugation. The supernatants obtained were stored at −20° C for further analysis. It has been shown that these procedures do not affect amino acid naphthylamidase in HeLa cells (*Lundgren* 1971 a).

The activities of amino acid naphthylamidase, acid phosphatase and alkaline phosphatase were assayed as described in the preceding paper (*Lundgren et al.* 1974). The effects on amino acid naphthylamidase activity of divalent metal ions, EDTA, the sulphydryl reagent p-OH-mercuribenzoate, Triton X-100 and puromycin were assayed as described in a previous publication (*Lundgren* 1971 a). In order to estimate substrate specificity the rate of hydrolysis of eight different amino acid naphthylamidases in 0.68 mM concentration was assayed in 0.05 M phosphate buffer pH 7.0. The pH dependence of the rate of hydrolysis was assayed with L-leucyl-β-naphthylamide as substrate with 0.05 M acetate buffer in the pH range 4.5–5.8, with 0.05 M Tris-maleate buffer in the pH range 5.3–8.5 and with 0.05 M Tris-HCl in the pH range 7.2–8.5. The K_m value was determined with L-leucyl-β-naphthylamide as substrate in the concentration range 0.5–0.01 mM at pH 7.0.

Polyacrylamide electrophoresis was performed and zymograms were obtained with L-alanyl-β-naphthylamide as substrate according to *Lundgren* (1971 a). Electrophoresis of the subfractions obtained after gradient centrifugation was performed in duplicate, the first run after freezing and thawing and the second run after Triton X 100 extraction as well.

Neuraminidase treatment was done according to *Spencer* (1970).

RESULTS

Electrophoresis

The extract obtained after freezing and thawing of the homogenate of the PMN preparation contained two components called isozymes A and B whereas that of the MN preparation contained only isozyme A (Fig. 1). In the Triton X 100 extracted material of the PMN preparation there was, besides B, a third component, called C, which was the only component of the MN preparation. The

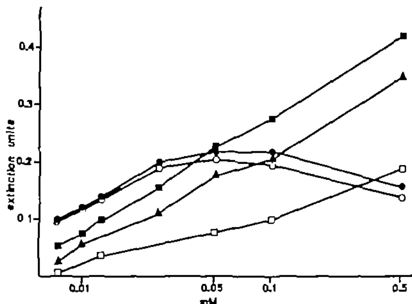


Fig 2 Relation between the concentration and cleavage of L-leucyl-β-naphthylamide incubated with fraction I (●) fraction II (■) and fraction III (▲) of the PMN preparation and fraction I (○) and fraction II (□) of the MN preparation.

integrity of the different isozymes was confirmed in mixing experiments.

If density equilibrium centrifugation of the 400 g supernatant of the PMN homogenate was performed in glycogen-sucrose gradients, three peaks with amino acid naphthylamidase activity were obtained which is in agreement with earlier results (*Lundgren et al* 1974) These peaks were interpreted to represent supernatant, low density granules and dense granules (fractions I–III respectively in figs. and tables) The subfractions of each peak were pooled and subjected to electrophoresis. In fraction I isozyme A dominated, but traces of isozyme B were found. Isozyme C was the only component in fraction II and isozyme B in fraction III When the MN preparation was used the same results were obtained with respect to fraction I and II but no more isozyme was found.

After treatment of fractions I–III with neuraminidase, only isozyme B was retarded on the gels which was taken as proof of available sialic acid residues, although the short migration distance of isozyme C excluded any conclusions regarding the sialic acid content of this isozyme.

Kinetics

K_M values were obtained by Lineweaver Burke plots. An apparent K_M value of 5–6×10^{-4} M was calculated for isozyme B and C of the PMN preparation and isozyme C of the MN preparation. The K_M value of isozyme A in both cell preparations was about 2×10^{-4} M. Isozyme A of both cell preparations was inhibited by high substrate concentrations (Fig 2)

pH-optimum

The pH-optimum was near neutrality in all three fractions extending from pH 6.0 to 8.0. The activity of isozyme A in the pH range of 7.2 to 7.8 was higher in Tris-HCl buffer than in Tris-maleate buffer whereas the activities of isozymes B and C did not differ (Table 1)

Substrate Specificity

Isozyme B and C in the PMN preparation and isozyme C in the MN preparation showed similar substrate specificity (Table 2) Both isozymes utilized the L-alanyl-derivate most efficiently followed by the L-leucine derivate Isozyme A, on the contrary utilized second

TABLE 1 *The Effect of Tris-Maleate and Tris-HCl Buffer on the Activity of Amino Acid naphthyl amidase Expressed as per cent of the Activity in Tris-Maleate Buffer pH 7.2*

pH value	Buffer	PMN			MN	
		Fraction I	Fraction II	Fraction III	Fraction I	Fraction III
7.2	Tris-maleate	100	100	100	100	100
	Tris-HCl	157	102	109	134	98
7.8	Tris-maleate	76	108	96	85	88
	Tris-HCl	134	88	96	123	75

Fractions I–III (for details, see text) were obtained from the PMN and MN preparation and corresponded to isozymes A, C and B respectively

TABLE 2. *Rate of Hydrolysis | Amino Acid β-N phthylamide Expressed as per cent of Maximal Activity*

Amino acid β-naphthylamide	PMN			MN	
	Fraction I	Fraction II	Fraction III	Fraction I	Fraction II
Alanine	100	100	100	100	100
Leucine	35	44	34	30	34
Isoleucine	1	6	26	0	4
Glycine	17	24	16	20	21
Arginine	67	18	20	60	28
Lysine	67	13	18	76	32
Proline	18	1	6	19	0

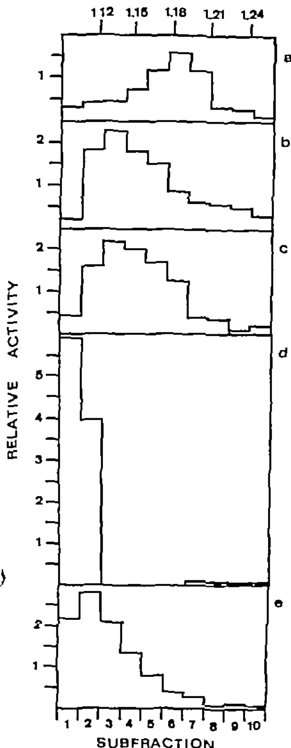
Fractions I–III (for details, see text) were obtained from PMN and MN leucocytes and corresponded to isozymes A, C and B, respectively

TABLE 3 *The Effect of Different Modifiers of Enzyme Activity Expressed as per cent of the cont ol A tivity*

Modifier		PMN			MN	
		Fraction I	Fraction II	Fraction III	Fraction I	Fraction II
Puromycin	1 mM	7	12	76	4	52
Triton X 100	1 per cent	27	100	81	17	50
p-OH-mercuri	0.1 mM	11	101	84	9	89
benzoate	0.01 mM	6	108	87		
EDTA	10 mM	2	12	26		
Co^{++}	1 mM	141	171	151	180	166
	0.1 mM	14	132	94	12	107
Cu^{++}	1 mM	5	9	28	4	14
	0.1 mM	4	39	40	6	43
Mn^{++}	1 mM	90	106	103	92	121
	0.1 mM	96	115	113	101	114

Fractions I III (for details, see text) were obtained from PMN and MN leucocytes and corresponded to isozymes A, C and B, respectively

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to the L-alanyl-derivate the β naphthylamides of the basic amino acids L-arginine and L-lysine more efficiently than L-leucine.

The Effect of Different Modifiers

Different substances with known selective effect on the isozymes of naphthylamidase were used (Table 3) Puromycin inhibited all isozymes, which indicated that these isozymes belong to the same class of mammalian naphthylamidase (*Lundgren 1971 a*) Triton X 100 inhibited isozyme A to the highest degree, whereas isozyme C of the PMN preparation was not affected Isozyme B of the PMN preparation and isozyme C of the MN preparation were inhibited to a moderate degree. p-OH mercuribenzoate inhibited selectively isozyme A, whereas isozymes B and C were affected only to a small extent. EDTA was strongly inhibitory to all isozymes. Co^{++} at 1 mM activated all isozymes whereas at 0 1 mM isozyme A was strongly inactivated and isozymes B and C were not essentially affected. Cu^{++} inactivated all isozymes and the inactivation of isozyme A was highest. Mn^{++} had little effect on all isozymes.

Among the modifiers, Triton X 100 was chosen to estimate the activity of different isozymes separately The activity resistant to 1 per cent Triton X 100 was used as an evaluation of isozymes B and C although it meant an underestimation of isozyme B of the PMN preparation and of isozyme C of the MN preparation (Table 3) The difference between the activity measured in the absence and presence of Triton X 100 was taken as an estimate of isozyme A.

Gradient Centrifugation

The 400 g supernatant of the MN and PMN homogenates was centrifuged in a sucrose-water gradient (Fig 3) The activity of

Fig 3 Sucrose-water density gradient subfractionation of the 400 g supernatant of the PMN (a-d) and MN (e) preparations. The relative activity is calculated as the enzyme activity present in each fraction divided by the calculated mean enzyme activity per fraction. a) acid phosphatase, b) alkaline phosphatase, c) Triton X 100 resistant amino acid naphthylamidase d) Triton X 100 sensitive amino acid naphthylamidase, e) Triton X 100 resistant amino acid naphthylamidase

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DISCUSSION

Three subcellular fractions enriched in amino acid naphthylamidase were demonstrated. Each fraction had a unique isozyme. Isozyme A seems to be soluble, whereas isozyme C is the isozyme of the suggested granules of low density (*Lundgren et al.* 1974). In the latter investigation, the third subcellular fraction could not be identified. In the present investigation, this fraction was found to contain isozyme B which co-sedimented with alkaline phosphatase. As reviewed by *Baggiolini* (1972) there is a discrepancy between the results obtained in cytochemical studies on the localization of alkaline phosphatase and the results obtained in cell fractionation studies. Thus, it is not known whether the granule of low density enriched in alkaline phosphatase really corresponds to the specific (or secondary) granules found by electron microscopy and cytochemistry.

Davies et al. (1970) reported that the bulk of amino acid naphthylamidase activity of rabbit polymorphonuclear leucocytes is separated from particles apparently identical with lysosomes. *Folds et al.* (1972) using human PMN leucocytes, found no activity in these granules but in granules of low density together with acid p-nitrophenyl phosphatase. *Lundgren et al.* (1974) confirmed that amino acid naphthylamidase of human leucocytes does not exist in lysosomes; on the other hand they could not establish that amino acid naphthylamidase belonged to the same fraction as acid p-nitrophenyl-phosphatase. As appears from that and the present investigation the activity can be resolved into three distinct isozymes, suggested to represent cytosol and two granules of different density one of which was enriched in alkaline phosphatase.

Isozyme A seemed to be distinct from isozymes B and C with respect to several biochemical parameters. On the other hand, isozymes B and C were very similar and differed only with respect to electrophoretic mobility, solubility properties and localization to subcellular components. Therefore they probably represent the same gene product and the differences noted could be due to a dual localization in the cell of one and the same enzyme molecule.

Naphthylamidases are supposed to be part of a sequence of proteolytic enzymes in digesting granules like lysosomes (*Tappel* 1969) hydrolyzing oligopeptides into single amino acids. Therefore it is remarkable that MN leucocytes seem to lack an isozyme of amino acid naphthylamidase in digesting granules. This observation may have some bearing on the known persistence of immunogenicity of microorganisms in MN leucocytes. An alternative possibility is that isozyme B is derived from isozyme C and appears in higher activity when the cell is phagocytosing or pinocytosing. However preliminary experiments have shown that no lysosomal naphthylamidases appear after phagocytosis of yeast cells.

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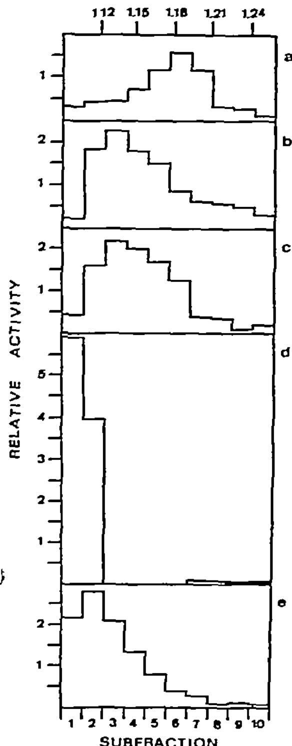


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ACUTE VIRAL HEPATITIS FACTORS POSSIBLY PREDICTING CHRONIC LIVER DISEASE

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Dietrichson, O Juhl, E., Christoffersen, P Elling, P., Faber V Iversen, K., Nielsen, J O Petersen, P & Poulsen, H. Acute viral hepatitis Factors possibly predicting chronic liver disease. Acta path. microbiol. scand. Sect. A, 83 183–188 1975.

A number of clinical, biochemical, immunological and morphological variables were recorded at first admission of 500 consecutive patients with biopsy verified acute viral hepatitis in the period February 1969–June 1972. In February 1973 28 of these patients had a morphologically documented chronic liver disease 4 cirrhosis of the liver 15 chronic aggressive hepatitis, and 9 chronic persistent hepatitis. 74 patients were followed up until morphological normalization took place. The initially recorded variables in the two groups were compared, and the following factors were significantly higher in the group with subsequent development of chronic liver disease —frequency of drug addicts, median of the highest gammaglobulin, ANA, SMA, partial destruction of the limiting membrane incidence of piecemeal necrosis, and pronounced plasma cell infiltration in the portal tracts. These preliminary results suggest that factors in the initial phase of acute viral hepatitis can be helpful to some extent in predicting the course and prognosis of the disease.

Key words Hepatitis, acut viral liver disease, chronic.

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The prognosis of acute viral hepatitis is regarded as good, and clinical and morphological normalization usually occurs (14)

However a correlation between acute viral hepatitis and chronic liver diseases has been demonstrated by means of serial liver biopsies (7 9 12 13)

It would be of great value if the prognosis of a case of acute viral hepatitis could be predicted in the early stage of the disease.

The present study consists of a comparison of the initially recorded variables in patients with uncomplicated acute viral hepatitis and those with subsequent development of chronic liver disease.

MATERIAL AND METHODS

500 consecutive patients with clinical and biopsy verified acute viral hepatitis admitted to the Department of Infectious Diseases, Blegdam Hospital and Medical Department 2, Kommunehospitalet, Copenhagen, during the period February 1969–June 1972 form the basis of the investigation.

A more detailed description of this material has already been published (11)

A number of clinical, biochemical immunological and morphological variables were recorded for

TABLE 1 *Clinical Aspects in Two groups of Patients with Acute Viral Hepatitis*

Clinical factors at first admission	Group with subsequent normalization n = 74		Group with subsequent chronic liver disease n = 28		Difference
	Per cent (95 per cent confidence limits)	Median (range)	Per cent (95 per cent confidence limits)	Median (range)	
Age (year)		22.5 (14–65)		20.5 (16–71)	n.s.
Women	41 (29–53)		39 (21–59)		n.s.
Anicteric cases	9 (4–19)		21 (8–41)		n.s.
Transfusions	3 (0.3– 9)		0 (0–12)		n.s.
Drug addicts (injections)	31 (?1–43)		61 (41–79)		p<0.05
Visits to endemic areas	32 (22–44)		14 (4–33)		n.s.
Hepatitis contact	46 (34–58)		64 (44–81)		n.s.
Liver enlargement	23 (14–34)		29 (13–49)		n.s.
Days in hospital		41 (2–193)		46 (1–164)	n.s.

n.s. not significant, i.e. p>0.05

all patients in the acute stage of the disease (11). The D (ad+ y—) and Y (ay+ d—) subtypes of Australia antigen were determined in cooperation with Dr G Le Bouvier Yale University as described in previous publications (8, 10).

The patients were controlled as far as possible after discharge and the intervals between the controls and their frequency were determined by the course of the disease and the patients cooperation. Clinical, biochemical and immunological investigations were made at each control, and it was planned to make a repeat biopsy three months after discharge and then each year.

In February 1973 74 patients had a resolution of the disease verified by biopsy. The mean follow up period for these patients has been 12.9 months (range 1–36 months). Twenty-eight patients had morphologically verified chronic liver disease i.e. 15 cases with chronic aggressive hepatitis, nine with chronic persistent hepatitis (duration of more than 6 months) according to criteria established by De Groote et al. (5) and four with cirrhosis of the liver. The mean period of observation in this group was 17.6 months (range 3–43 months). These 102 patient (20.4 per cent) were followed-up until morphological normalization or the development of chronic liver disease had taken place.

Two patients died in the acute stage of the disease one of acute liver failure and the other of a pulmonary disease.

Of the remaining 396 patients, 191 still had changes according to the criteria for acute viral hepatitis (3) in the last repeat biopsy and the mean morphological follow-up period was 4.8 months (range 1–29 months). 205 patients refused repeat biopsy or could not be traced for follow-up.

A comparison was made of the initially recorded clinical, biochemical, immunological and morphological variables in the two well-defined morphological groups, viz. 74 patients with normalization and 28 patients with development of chronic liver disease.

The non-parametric Mann-Whitney test was used in the statistical analysis (1). The quantitative variables are recorded by median and range. Confidence limits for the binominal distribution are from Geigy Scientific Tables.

RESULTS

Clinical Findings

The incidence and medians of some clinical and anamnestic variables are shown in Table 1. The only significant difference between the two groups of patients was in the incidence of drug addicts (injections) which was highest in the group with subsequent development of chronic liver disease.

Biochemical Findings

The medians of some conventional liver tests at the time of diagnosis and the highest or lowest values of these during the first stay in hospital are shown in Table 2.

There are some differences, but only one of these is significant, viz. the median of the highest registered bilirubin value which was

TABLE 2. *Biochemical Liver Tests in Two Groups of Patients with Acute Viral Hepatitis*

ochemical factors first admission	Group with subsequent normalization n = 74		Group with subsequent chronic liver disease n = 28		Differences
	Median	(range)	Median	(range)	
partate transaminase (10–25 u/l)	318	(29–5755)	500	(53–2668)	n.s.
ighest transaminase	850	(29–5756)	952	(49–2795)	n.s.
lkaline phosphatase (30–90 u/l)	124	(47–441)	159	(58–447)	n.s.
ighest alkaline phosphatase	144	(47–517)	154	(61–763)	n.s.
ilirubin (<1.0 mg/100 ml)	4.9	(0.5–2_.0)	2.5	(0.5–15 7)	n.s.
ighest bilirubin	8.0	(0.5–59.0)	4 1	(0 5–22.4)	p<0.05
othrombin (>70 per cent)	70	(18–156)	59	(18–25)	n.s.
owest prothrombin	56	(18–150)	50	(14–88)	n.s.
bumin (4 4–5.9 g/100 ml)	4.3	(3.2–6.3)	4 1	(3.3–5 1)	n.s.
owest albumin	4.2	(3.0–5 4)	4.0	(3 1–5.1)	n.s.

n.s. not significant, i.e. $p > 0.05$.
Normal values given in brackets under laboratory tests.

highest in the group with subsequent morphological normalization.

Immunological Findings

The incidence and medians of some serological factors are shown in Table 3. The difference between the two groups as regards the highest gammaglobulin values during the first stay in hospital was significant, with the highest values in the group with subsequent development of chronic liver disease. The incidence of antinuclear antibodies (ANA) and smooth muscle antibodies (SMA) was significantly higher in the same group. Australia (Au) antigen also occurred more frequently in the "chronic" group, but this difference was not significant. A statistically significant difference was found in the distribution of subtypes, with a higher incidence of subtype Y in the group with development of chronic liver disease as compared to the group with morphological normalization.

Morphological Findings

Tables 4 and 5 show some portal and parenchymal changes found in the initial biopsy.

There was a significantly higher incidence of cases with defect limiting plate piecemeal necrosis and pronounced plasma cell infiltration in the portal tracts in the group with subsequent development of chronic liver disease than in the group with normalization.

DISCUSSION

The prognosis in cases of acute viral hepatitis is usually good (14). In the present study of 500 consecutive biopsy verified patients, a follow-up study revealed 28 patients with morphologically documented chronic liver disease, i.e. 5.6 per cent (95 per cent confidence limits 3.8–8.0). If the complication cirrhosis of the liver is regarded alone, the incidence was 0.8 per cent. This is not a real incidence but an absolute minimum, since only about one fifth of the material has been followed up to the morphological stages chronic liver disease or normalization. In addition, some patients were observed for less than one year and some of them still had morphological changes compatible with acute viral hepatitis. However there is reason to believe that patients with a complicated course of the disease participated in the con-

TABLE 3 *Immunological Findings in Two Groups of Patients with Acute Viral Hepatitis*

Immunological factors at first admission	Group with subsequent normalization n = 74		Group with subsequent chronic liver disease n = 28		Differen…
	Per cent (95 per cent confidence limits)	Median (range)	Per cent (95 per cent confidence limits)	Median (range)	
IgG (7.2–15 1 g/l)		14 1 (6.1–24.8)		15.5 (11.3–29 7)	n.s.
IgA (0.74 3 06 g/l)		1.55 (0 40–3 65)		1.51 (0.53–2.54)	n.s.
IgM (0.25–1.35 g/l)		0.92 (0.30–4 47)		0.89 (0.36–3.63)	n.s.
Highest gammaglobulin g/l		13 6 (7.2–35.5)		16.3 (10 0–44.0)	p<0.0[illegible]
Antinuclear antibodies	7 (2–15)		25 (11–45)		p<0.0[illegible]
Waaler–Rose	0 (0– 5)		4 (0 1–18)		n.s.
Mitochondrial antibodies	2 (0.3– 9)		0 (0–12)		n.s.
Smooth muscle antibodies	26 (16–37)		54 (34–72)		p<0.0[illegible]
Australia antigen	46 (34–58)		61 (41–79)		n.s.
subtype Y	24 (15–36)		57 (37–76)		p<0.0[illegible]
subtype D	16 (9–27)		4 (0.1–18)		p<0.0[illegible]
subtype undetermined	6 (2–13)		0 (0–12)		n.s.
Blood group A	43 (32–55)		54 (34–37)		n.s.
Blood group B	8 (3–17)		4 (0.1–18)		n.s.
Blood group AB	4 (0.8–11)		0 (0–12)		n.s.
Blood group O	45 (33–57)		42 (24–63)		n.s.
Rhesus positive	82 (72–90)		98 (72–98)		n.s.

n.s. not significant, i.e. $p>0.05$ Normal values given in brackets after the tests.

TABLE 4 *Portal Changes in Liver Biopsies from Two Groups of Patients with Acute Vi al Hepatitis*

Portal changes at first admission	Group with subsequent normalization n = 74				Group with subsequent chronic liver disease n = 28				Differenc…
	number +	++	+++	per cent (95 per cent confidence limits)	number +	++	+++	per cent (95 per cent confidence limits)	
ls	25	0	0	34 (23–46)	15	1	0	57 (37–76)	n
ls in portal macrophages	22	12	1	47 (36–59)	5	1	1	25 (11 45)	n.s.
ilic granulocytes	31	0	0	42 (31–54)	14	1	1	57 (37–76)	n.s.
ocytes		18	0	24 (15–36)		11	0	39 (22 59)	n.s.
sma cells		9	0	12 (6–22)		12	1	46 (28–66)	p<0 001
Eosinophilic granulocytes	26	1	0	36 (26–48)	9	1	0	36 (19 56)	n.s.
Other leucocytes	40	26	0	89 (80–95)	11	9	0	78 (72 98)	n.s.
Bile duct proliferation	15	0	0	20 (12–31)	7	0	0	25 (11–43)	n.s.

+ slight, + + moderate + + + severe. n.s. not significant, i.e. $p>0.05$

trol to a considerable extent and agreed to repeat biopsy in contrast to the patients with a benign, uncomplicated course

In a partially selected material, nine cases of cirrhosis occurred in a follow up study of 120 cases with acute viral hepatitis (13)

It would be of great importance if patients with subsequent development of chronic liver disease could be diagnosed already in the initial phase of acute viral hepatitis. This small group should be controlled regularly and treatment with steroids or other im-

TABLE 5. *Parenchymal Changes in Liver Biopsies from Two Groups of Patients with Acute Vi al Hepatitis*

enchymal changes first admission	Group with subsequent normalization n = 74 number +	++	+++	per cent (95 per cent confidence limits)	Group with subsequent chronic liver disease n = 28 number +	++	+++	per cent (95 per cent confidence limits)	Differences
ect limiting plate	14	0	0	19 (11–30)	14	0	0	50 (31–69)	p<0.05
al necrosis		17	0	23 (14 34)		4	0	14 (4–33)	n.s.
ce meal necrosis	20	1	0	29 (19–40)	12	3	0	54 (34–72)	p<0.05
ration of nuclei		28	0	38 (27–50)		7	0	25 (11–45)	n.s.
dation of cells		48	2	68 (56–78)		18	1	68 (48–84)	n.s.
dophilic bodies		15	1	22 (13–33)		5	0	18 (6–37)	n.s.
massive necrosis	7	1	0	11 (8–25)	5	1	0	21 (8–41)	n.s.
n in Kupffer cells	21	12	8	55 (43–67)	9	3	0	43 (24–63)	n.s.
nulocytes	3	0	0	4 (0 8–11)	0	0	0	0 (0–1)	n.s.
rafocytes	31	3	0	46 (34 58)	18	0	0	64 (44–81)	n.s.
uma cells	27	2	0	39 (28–51)	9	2	0	39 (22–59)	n.s.
rpffer cell proliferation		8	1	39 (28 51)		15	1	57 (37–76)	n.s.
olestasis	10	6	0	22 (13 33)	4	1	0	18 (6–37)	n.s.

+ slight, + + moderate, + + + severe. n.s. not significant, i.e. p>0 05

munosuppressive drugs may be considered in this category of patients.

The present work is a comparison of the initial variables in patients with acute viral hepatitis with both complicated and uncomplicated course revealed by means of serial liver biopsies. Because of the poor possibility of follow up and probably also because of the usually benign course of the disease, the group of patients with chronic liver disease is small. Therefore patients with morphologically diagnosed cirrhosis, chronic aggressive hepatitis, and chronic persistent hepatitis have been assembled in one group. It is realized of course, that the prognosis of the latter disease is generally regarded as good and that cirrhosis apparently develops only rarely in this condition (2, 4)

As the number of patients in both groups is limited, the significant differences between them are few. If complete follow-up of the material had been possible other differences might have been significant.

The majority of drug addicts were found in the group with development of chronic liver disease. None of the drug addicts had biopsy documented cirrhosis but either chronic aggressive or chronic persistent hepatitis.

The biochemical variables were of no prognostic help. Only in the highest registered bilirubin values was there a significant difference, the highest values being in the group with later normalization. It must be taken into consideration that more anicteric cases occurred in the "chronic" group with many drug addicts. Because of the frequent use of contaminated syringes and needles, these patients were often detected as anicteric cases by routine blood controls.

As regards the immunological factors, differences were fond in the incidence of circulating auto-antibodies (ANA and SMA) and in the distribution of Au-antigen subtypes. It was demonstrated in a previous publication that patients with chronic *hepatitis* and ANA and/or SMA but without Au-antigen had a poor prognosis as compared to patients with the same morphological changes and positive reaction for Au-antigen but without circulating auto-antibodies (6). The present study shows that the incidence of ANA and SMA in the acute phase of acute viral hepatitis is significantly higher in patients with subsequent development of chro-

nic hepatitis or cirrhosis than in patients with an uncomplicated course of the disease.

It has been reported previously that there is a nonsignificant predominance of the Y subtype in patients with progression from acute to chronic hepatitis, as compared to patients with complete resolution (10) The difference has now reached the 0 05 significance level, but it should be emphasized that this difference may be due to a possible selection in the material rather than to biological differences between the two subtypes. Nearly all the drug addicts had antigen of the Y subtype (98 per cent) and it seems reasonable to postulate that the difference reflects the difficulty in performing late follow-up biopsies in drug addicts with complete resolution.

Comparison of the morphological changes reveals differences in some of the factors used for characterizing chronic hepatitis (5) These changes occurred most frequently in patients with subsequent development of chronic liver disease. In this context it must be emphasized that all patients fulfilled the morphological criteria for acute viral hepatitis at the time of investigation (3 11)

The results of the study indicate that some factors in the initial stage of acute viral hepatitis may be of prognostic help. Already initially there seem to be differences between cases with subsequent development of chronic hepatitis and those with a benign course of the disease.

Both the immunological and the more detailed morphological changes may indicate that autoimmune mechanisms may be of importance in the various reactions and course. The changes which distinguish the two groups at the start of acute viral hepatitis are to some extent. the same as those which are of importance in predicting the prognosis of chronic hepatitis.

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BRIEF REPORTS

PROLIFERATING AND NON-PROLIFERATING COMPARTMENTS IN CERVICAL DYSPLASIA AND CARCINOMA *IN SITU*

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Rubio, C. A. & Lagerlöf, B. Proliferating and non-proliferating compartments in cervical dysplasia and carcinoma *in situ*. Acta path. microbiol. scand. Sect. A, 83: 189–191, 1975.

The existence of proliferating and non-proliferating compartments in atypical cervical epithelium is suggested by autoradiographical investigations and studies on the topographical distribution of mitoses performed on human cervical cones and on cervices of mice following carcinogenic treatment.

Key words: Carcinoma in situ; cervical dysplasia; cell proliferation.

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During analysis of the frequency of cells in DNA synthesis in dysplasia and carcinoma *in situ* in human cervical cones (*Rubio & Lagerlöf* 1974) and in corresponding intra-epithelial atypias in the cervix of mice (*Rubio & Lagerlöf* 1974b) it was observed that the H-thymidine-labelled cells were often distributed unevenly in the epithelium. Areas of labelled cells alternated with autoradiographically negative areas (Fig. 1). The latter areas remained unlabelled even after 2 hours incubation *in vitro*.

During these studies it was apparent also that even the dividing cells adopted an uneven distribution. These observations led us to investigate in detail the topographical distribution of mitotic figures in cervical punch biopsies and subsequent cone specimens in 7 cases of carcinoma *in situ*. A total length of 3,200 μ of epithelium with carcinoma *in situ* were investigated in each case. The results demonstrated that areas rich in dividing cells (Fig. 2) alternated with areas devoid of mitotic figures (Fig. 3) in many areas of the epithelium presenting the same histological alteration.

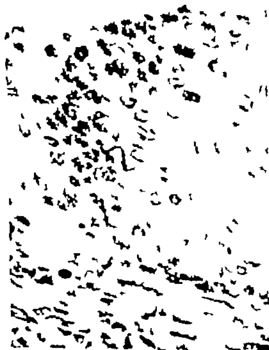


Fig. 1. Area of carcinoma *in situ* with a high number of H-thymidine labelled cells. Note unlabelled adjacent areas (× 400).

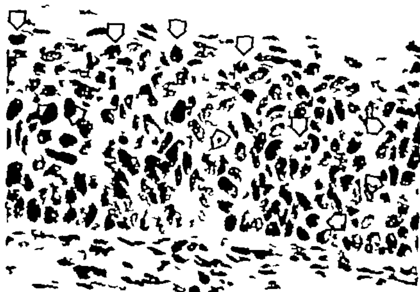


Fig. 2. Area of carcinoma *in situ* with a high number of mitotic figures (arrows). (× 400)



Fig. 3. Another area in the same case as in Fig. Note absence of mitoses. (× 400)

After injection of colchicine into 83 mice with a view to arresting mitosis it was found that benzpyrene-induced intra-epithelial atypias also have areas rich in mitoses alternating with areas in which dividing cells are lacking (*R blo & Lagerlöf* in preparation).

The autoradiographical findings as well as the topographical distribution of mitosis suggest the existence both in man and in mouse of proliferating and non-proliferating (i.e. resting) compartments in the cervical epithelium containing intra-epithelial atypias. Proliferating and non-proliferating cell compartments have been reported earlier by *Mendelsohn* (1962) and by *Post et al.* (1973) to be in evidence in solid tumours of experimental animals following incorporation of ^{3}H-thymidine.

Some questions remain to be solved before a more informative picture of the phenomenon described above can be obtained, for instance how long the cell remain in the proliferating and resting compartments, respectively, and which are the

factors that regulate the possible alternating epithelial proliferation in cervical atypias.

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ENDOCRINE CELLS IN THE KIDNEY

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There is a series of cells in the body that have been shown to have amine-handling properties and at the same time secrete polypeptide hormones. This series has been reviewed by *Pearse & Polak* (1974) under the common name of the APUD-cell series. Although the APUD system has not been described in the kidney (*Pearse & Polak* 1974) the known hormonal role of the kidney prompted a search for cells of the APUD system. The results of a preliminary study will be reported here.

Material and Methods

Kidney tissue from 3 humans (both adult and paediatric) guinea-pigs, rats (Wistar) and mice (C3H) was studied. Samples from adrenal, duodenum and pancreas from guinea-pigs, rats and mice were used as control. These samples were fixed in potassium dichromate-chromate for 48 hrs and buffered neutral formalin 10 per cent for 24 hrs, formalin calcium-chloride for 72 hrs, Bouin (1 per cent acetic acid) for 24 hrs, 6 per cent buffered glutaraldehyde, 6.5 per cent glutaraldehyde in Millonig buffer at pH 7.3 for 12 hrs, and glutaraldehyde-picric acid (*Solcia et al.* 1968).

The chromated specimens were studied with the chromaffin-formalin-nuclear fast red sequence (*Falck et al.* 1959) in frozen sections. The glutaraldehyde-silver method for adrenaline and noradrenaline (*Tramezzani et al.* 1964) was carried out in rat adrenals and kidneys fixed in 6.5 per cent glutaraldehyde. The others were washed 6 hrs in running water 24–48 hrs in 70 per cent ethanol, dehydrated and embedded in 56 C paraffin wax.

Sections (5 µ) from all fixatives were stained with haematoxylin-eosin, Bouin fixed with argyrophil method of Grimelius (1968) formalin calcium-chloride fixed with argentaffin method of Masson-Fontana (*Pearse* 1972) ferri-ferricyanide reduction test (*Adams* 1956) and sections examined in water previous to counterstain, Perl' method for iron (*Pearse* 1972) long Ziehl-Neelsen (*Pearse* 1972) diazonium reaction with fast Garnet G.B.C., periodic acid Schiff (PAS) p-dimethylaminobenzaldehyde (DMAB)-nitrite method (*Adams* 1957) and toluidine blue 1 per cent, pH 5.

Glutaraldehyde fixed with diazonium reaction with fast Garnet G.B.C. and fast red salt B (*Lillie & Glenner* 1960) p-dimethylaminobenzaldehyde (DMAB)-nitrite method, xanthydrol method (*Lillie* 1957) azure A and toluidine blue after acid hydrolysis (0.2N hydrochlorid acid at 60° C for 3 hrs) for masked metachromasia (*Solcia et al.* 1968) and lead haematoxylin method for endo-

crine granules after acid hydrolysis (0.2N hydrochloric acid at 60 C for 8 hrs) (*Solcia et al.* 1969) Glutaraldehyde-picric acid fixed with the same method for masked metachromasia after 3 and 10 hrs of acid hydrolysis and the same method for lead haematoxylin.

Results and Comments

The presence in the kidney of 2 separate series of cells was demonstrated. Both were well developed in man and rat, and less so in guinea-pig and mouse. Both were located in the cortical and juxtamedullary area of the kidney of all studied species. They consisted of a tubular system with chromaffin characteristics and a paratubular system with APUD features.

The *tubular system* consisted of granulated cells with the following histochemical characteristics presence of chromaffin granules negative for dopamine (*Falck et al.* 1959) argyrophilia both in granulated and non-granulated cells (*Grimelius* 1968) argentaffinity (Masson-Fontana) and positive ferri-ferricyanide reduction test.

The combination of stains used here are those used by *Solcia et al.* (1969) to demonstrate the presence of biogenic amines in glutaraldehyde fixed material. The diazonium reaction was positive in the granules of the tubular cells while the xanthydrol and DMAB-nitrite method were negative. Therefore as the granules were negative for dopamine, the probable amine content of this chromaffin tissue could be nor-adrenaline. This was further indicated by a positive glutaraldehyde-silver method.

Morphologically similar granules stained yellow brown with haematoxylin-eosin negative with PAS and Perl's methods and occasionally positive with the long Ziehl-Neelsen method. These granules drew the attention of *Sacco et al.* (1958) who studied the pigment histochemically in the proximal convoluted tubules of the kidney of a type of snake. They demonstrated the existence of 2 pigments an argentaffin type represented the greater quantity and small amounts of lipofuchsine type. *The presence of argentaffin granules with arylamine content were also reported by Mukherji &* Sen (1964) in proximal convoluted tubule of toad kidneys.

The *paratubular system* showed the following histochemical characteristics argyrophilia, positive masked metachromasia and basophilia, negative diazonium reaction, occasional argentaffinity and positive ferri-ferricyanide reduction test characterized by blue colour and tryptophan content.

The argyrophilia was demonstrated with Grimelius and the argentaffinity with Masson-Fontana. The diazonium reaction in formalin calcium-chloride fixed material was negative, but the DMAB-nitrite method was positive which indicates tryptophan content. Following the method of *Solcia et al.* (1969) the different reactions for "phenol" and "indole" methods in glutaraldehyde fixed material were studied. With this fixative the DMAB-nitrite gave a blue reaction while that of xanthydrol was purple-violet confirming the tryptophan content of the cells. *In this material the diazonium reaction* was also negative eliminating the possibility of the presence of 5-hydroxytryptamine. The paratubular cells showed masked metachromasia both in glutaraldehyde and glutaraldehyde-picric acid fixed material after 3 and 10 hrs hydrolysis. The best result was given by toluidine blue after 3 hrs hydrolysis. Serial sections with toluidine blue 1 per cent, pH 5 failed to stain the paratubular cells but showed a few mast cells in the perirenal tissue. The results with lead haematoxylin were similar to those with masked metachromasia in the best and in quality. This was interpreted as a result of a short time of acid hydrolysis.

The histochemical characteristics of these cells are thus similar to those of a type of endocrine cell described in the gastrointestinal tract (*Fiocca et al.* 1969).

The present study demonstrates therefore that the kidneys in man, rats, guinea-pigs and mice contain a tubular system with chromaffin characteristics and a paratubular system with APUD characteristics.

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PLASMINOGEN ACTIVATORS IN OVARIAN TUMOURS

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Svanberg, L., Linell, F., Pandolfi, M. & Astedt, B. Plasminogen activators in ovarian tumours. Acta path. microbiol. scand. Sect. A 83: 193–198, 1975

Ovarian tumours obtained at laparotomy were histochemically examined for their local fibrinolytic activity and simultaneous fibrin/fibrinogen degradation products (FDP) were determined in the serum. The fibrinolytic activity was confined mainly to vessels of both malignant and benign tumours. A very close correlation was demonstrated between the fibrinolytic activity and the vascularity of the sections. FDP were found in the serum in 13 of 14 patients with malignant tumours, but in none with benign tumours. The difference in occurrence of FDP in patients with malignant and benign tumours might be due to the invasive growth of the former with the entrance of thromboplastic substances, fibrinolytic activators or locally formed FDP into the bloodstream.

Key words: Ovarian tumours; plasminogen activators.

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Fibrin/fibrinogen degradation products (FDP) occur in the serum of patients with various malignant diseases (*Merskey et al.* 1966, *Colman* 1970, *Hedner & Nilsson* 1971). Such products have been demonstrated in 72 per cent of the sera from patients with malignant ovarian tumours (*Åstedt et al.* 1971) and invariably and in high concentration in malignant ascitic fluid (*Åstedt et al.* 1972, *Svanberg et al.* 1974). FDP occur in the blood in the presence of conditions with associated general fibrinolysis, intravascular coagulation with secondary fibrinolysis or local deposition of fibrin with secondary fibrinolysis. Thus an indispensable condition for the appearance of FDP is fibrinolytic activity.

In an endeavour to shed further light on the role played by the local fibrinolytic activity of tumours in the development of FDP we histochemically assessed the fibrinolytic activity and the vascularization in a series of ovarian tumours and the simultaneous amount FDP, if any, in the serum.

MATERIAL AND METHODS

The material consisted of biopsy specimens of 25 fresh ovarian tumours and of 14 normal ovaries, obtained at laparotomy of non-irradiated patients. In one patient, biopsy specimens were obtained both before and after irradiation. Specimens of the ovarian tumours and normal ovaries were immediately frozen in expanding CO_2 to prevent freezing artefacts. They were packed hermetically in metallic foil to prevent them from drying and were stored at −70° C until examined.

Blood samples for determination of FDP were collected just before laparotomy

The *fibrinolytic activity* was determined histochemically using the method of *Todd* (1959) as modified by *Pandolfi* (1972) The specimens were cut on an International Harris cryostat in sections 8 μ thick and collected on cleaned glass slides. Four sl des were prepared for each sample. The sections on each slide covered with 0.06 ml of fibrinogen (bovine fibrinogen prepared essentially according to *Brakman s* modification (1967) of *Astrup & Müllertz* (1952) double ammonium sul phate precipitation method) in a concentration of 1 per cent in phosphate buffer ph 7.8 (ionic strength 0 15) and of 10 μl thrombin (Topostasin 20 NIH units/ml unbuffered saline) The fibrinogen-thrombin mixture was spread over an area of 10 mm² in order to obtain a fibrin film about 0.07 mm thick. To stabilize the fibrin film, the slides were left at room temperature (21–24 C) in a moist chamber for 30 minutes. The slides were transferred to another moist chamber at 37 C and incubated for 15 30 and 45 minutes, respectively after which they were fixed in forma lin. The slides were stained with Giemsa solution and Harris' haematoxylin. Fibrinolysis was re flected by clear lytic areas in the fibrin film at the site of fibrinolytically active cells. Three fairly distinct grades of fibrin digestion were recognized, namely grade I microscopical punctate areas in most of the sections, grade II: gross lytic areas of irregular outline and sometimes confluent, grade III dissolution of most or all the fibrin in contact with the sections.

The vascularization of the specimens, i.e. number and size of essels, was graded as poor (+) moderate (+ +) or well (+ + +)

The occurrence of fibrin d posits and necroses in the tumours was determined from the histological slides, mostly 10 or more from every tumour

Fibrin/fibrinogen degradation products (FDP) Blood was collected in tubes containing EACA (epsilon aminocaproic acid) and thrombin (10 NIH units thrombin and 25 mg EACA to 3 ml blood) Serum from these samples was prepared in the way described by *Nilehn* (1967) FDP were determined by the immunochemical method of *Nilehn* (1967) In this method an antiserum against the D fraction of the FDP is applied to agarose gel. At high voltage electrophoresis, serum, or the medium to be tested, migrates into the gel. If FDP (X Y or D-products) are present, they will produce precipitation peaks. The height of such peaks is measured and related to a standard of substances of high molecular weight. The sensitivity of the method is 5 μg/ml. In the presence of EACA and thrombin this method will not show any FDP in the serum of healthy control women, even in repeated assays.

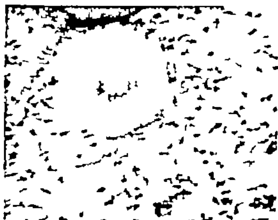


Fig 1 Not Fibrinolytic zones confined to the small essels. × 25

RESULTS

The fibrinolytic activity in malignant and benign ovarian neoplasms as well as in normal ovaries was found to originate from vessels (Fig 1) This activity was closely correlated to the vascularity of the areas studied (Fig 2A and B Fig. 3A and B) No correlation was found between fibrinolytic activity and malignancy (Table 1)

FDP were found in the serum in 13 of 14 patients with malignant tumours, but in none of those with benign tumours. Necrosis and fibrin deposits were found in most of the malignant tumour specimens (Table 1)

In the additional case, in which tumour specimens were examined before and after irradiation the fibrinolytic activity of the tumour was no longer demonstrable after radiotherapy At the same time the concentration of FDP in the serum and of that in the ascitic fluid were decreased (Table 2)

DISCUSSION

The fibrinolytic activity of malignant as well as benign tumours was found to originate from vessels. This is in agreement with observations by *Weiss & Beller* (1969) in uterine tumours and by *Pick et al* (1971) in experimental tumours. The fibrinolytic activity was thus found to be correlated to vascularization and not to malignancy

TABLE 1 *The Fibrinolytic Activity, Degree of Vascularization and Necrosis and Fibrin Deposits in Primary Ovarian Neoplasms (F.I.G.O. Classification) and FDP in the Serum*

Type of tumour (n = 25)		Fibrinolytic activity (arbitrary units)	Degree of vascularization	Necrosis and fibrin deposits	FDP/serum (μg/ml)
I. Neoplasm with endocrine significance	(n = 1)	8	+ +	yes	29
II. Neoplasm with no endocrine significance					
a. germ cell tumours	(n = 3)	8.5	+ +	no	25
		9	+ + +	yes	20
		5.5	+	yes	10
b. ovular tumours	(n = 1)	8	+ +	no	0
c. stromatogenous tumours	(n = 3)	5	+	no	0
		8.5	+ +	no	0
		9	+ + +	no	0
d. epithelial tumours					
serous (1A)	(n = 3)	4	+	no	0
		8	+ +	no	0
		5.5	+	no	0
serous (1B)	(n = 2)	11.5	+ + +	no	0
		11.5	+ + +	no	0
serous (1C)	(n = 6)	4	+	no	10
		8	+ +	yes	10
		7.5	+ +	yes	33
		3	+	yes	34
		1.5	+	yes	15
		4.5	+	yes	30
mucinous (2A)	(n = 2)	10.5	+ + +	no	0
		10	+ + +	no	0
mucinous (2C)	(n = 2)	8	+ + +	yes	0
		12	+ + +	no	22
III. Ovarian metastases	(n = 2)				
from colon carcinoma		4.5	+	yes	25
from gall bladder carcinoma		11	+ + +	yes	15

\+ = poorly vascularized.
\+ + = moderately vascularized.
\+ + + = well vascularized.

TABLE 2. *Local Fibrinolytic Activity and FDP Before and After Radiotherapy (a Patient with Ovarian Concomitant Carcinoma (4C)*

	Activity in arbitrary units	FDP/ in μg/ml	FDP/ascites in μg/ml
Before radiation	3.5	35	1200
After radiation	0	15	0

The finding of FDP in most of the patients with malignant tumours and in none of the patients with benign tumours is in accord with earlier observations by *Astedt et al.* (1971).

The main purpose of the study was to elucidate the role played by the local fibrinolytic activity of the tumours in the occurrence of FDP.

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Fig 2A Cortical area of ovary × 16. Arbitrary units 3



Fig 2B Central area of ovary × 16. Arbitrary units 10.5.

Vascularity of cortical and central areas of ovary To the left in Fig 2A cortical stroma with few and thin vessels. To the left in Fig 2B in contrast a lot of vessels in the central part.

be difference in the occurrence of FDP he serum in malignant and benign cases ht be explained by a certain degree of ral fibrinolysis due to the invasive growth he malignant tumours with easier escape brinolytic activators into the bloodstream. nother explanation might be the passage romboplastic substances (*O'Meara* 1958, & *Jancer* 1968, *Tori et al.* 1974) the bloodstream with intravascular coa tion and secondary fibrinolysis as a re Disseminated intravascular coagulation associated bleeding tendency has been rted by *Straub* (1971) and *Samaha et al* 3) in patients with metastasing tumours. it the occurrence of FDP in the serum might also be due to the entrance of locally formed FDP into the bloodstream. Such possibility requires the assumption of the presence of local fibrinolytic activity as well as of local fibrin deposits. This requirement was met in most of the malignant tumours in which not only fibrinolytic activity but also necroses and fibrin deposits were found.

After radiotherapy FDP in blood and ascitic fluid decreased and disappeared (*Stra-berg et al.* 1973) This occurred also in the additional case In that case the fibrinolytic activity of the tumour vessels also disappeared, thereby stressing the significance of the local fibrinolytic activity for the occurrence of FDP

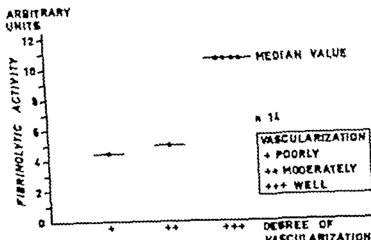


Fig. 3A. Fibrinolytic activity in poorly moderately and well vascularized normal ovaries. (Arbitrary units. —— denotes median value)

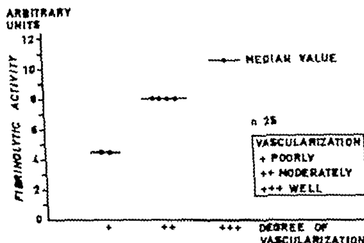


Fig. 3B. Fibrinolytic activity in poorly moderately and well vascularized ovarian tumours. (Arbitrary units. —— denotes median value)

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INFLUENCE OF NEONATAL THYMECTOMY ON BLOOD PRESSURE AND HYPERTENSIVE VASCULAR DISEASE IN RATS WITH RENAL HYPERTENSION

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Svendsen, U. G. Influence of neonatal thymectomy on blood pressure and hypertensive vascular disease in rats with renal hypertension. Acta path. microbiol. scand. Sect. A, 83: 199–205, 1975.

The effect of neonatal thymectomy on the degree and prognosis of hypertension and on the vascular lesions in rats with renal hypertension was studied. There were no differences between thymectomized and sham operated hypertensive rats. The degree of hypertension, the frequency of spontaneous death and heart infarcts were the same in both groups. The occurrence and degree of perivascular cell infiltrations, deposits of perivascular connective tissue and fibrinoid degenerations of the media were found to be the same in both the thymectomized and the sham operated hypertensive animals. The results do not support the assumption that delayed type immune reactions are important in the pathogenesis of hypertensive vascular disease in renal hypertensive rats.

Key words: Thymectomy; neonatal; blood pressure; hypertensive vascular disease; rats.

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Hypertensive vascular disease is characterized by thickening and degeneration of the intima, hyperplasia, and in more pronounced cases "fibrinoid" necrosis of both intima and media, and adventitial cellular reaction, including mononuclear inflammatory cells and fibroblasts (for literature see *Pickering* (1968) and *Olsen* (1971)). The cause of the intra- and perivascular inflammatory cellular reaction can either be a secondary reaction to the vascular damage or a sign of an immunological reaction which may be a pathogenic factor for the vascular lesion and thus perhaps also for the hypertension (7–10).

The adventitial cellular infiltration has a similarity to that seen in delayed type immune reactions, for which the thymus plays a role (4, 12). The aim of the present work has been to study the pathogenic role of immune reactions for both hypertension and vascular disease in rats with experimental *Goldblatt* hypertension. For this purpose rats were neonatally thymectomized and subsequently made renal hypertensive by means of a Goldblatt clip. The degree of hypertension and of hypertensive vascular disease,



Fig 1 Arteries from the heart of renal hypertensive neonatally thymectomized rats showing a normal round cell infiltration in adventitia (a, 1+) and a 3+ round cell infiltration in b. A total fibrinoid necrosis of media is apparent in b. Magnification 140 × PAS stain.

and especially the cellular reaction, in thymectomized and sham operated littermates were compared

MATERIAL

Animals SPF outbred Wistar female and male rats from Møllegaard Hansens Breeding Laboratories A/S were fed rat pellets and received tap water ad libitum.

METHODS

The experimental animals were divided into groups as follows:

Group I 27 neonatal thymectomized rats with Goldblatt clip on the left artery 8 males and 19 females. Litters were *thymectomized* during the first 24 hours after birth. Anaesthesia was induced by cooling for 15 minutes in the refrigerator An incision was made in the midline and the sternum was cut to the level of the second costa. The pretracheal muscles were split in the midline and the cranial parts of the thymus lobes were freed from the underlying tissue The thymus was sucked out through a glass tube. After complete removal of the thymus the incision was closed with silk sutures in the skin.

A Goldblatt silver clip was applied on the left renal artery under ether anaesthesia when the rats were 6 weeks old The internal diameter of the clip was 0 20 mm The kidney was placed subcutaneously Following the operation all rats received 0.1 ml Dipenicillin® Leo subcutaneously It was attempted to allow the animals to live for 5 months after clipping the renal artery

3 months after application of the clip blood pressure was determined every second day for a period of 2–3 weeks in the conscious animals. A tail plethysmograph method was used (16) 7 rats had their blood pressure recorded for a second period of 2 weeks, 30 days after the first period,

After sacrifice or spontaneous death the heart, the kidney and the pancreas were fixed in 4 per cent formalin and embedded in paraffin. Sections 5 microns thick were cut and stained with Van Gieson Hansen (VGH) periodic acid Schiff (PAS) and haematoxylin eosin (HE) stains. The round cell infiltrations around arteries in the heart, the arcuate/interlobular arteries of the kidney and arteries in the pancreas were graded semiquantitatively according to a photographed scale from 1+ to 3+ in which a 1+ cellular reaction includes the sparse mononuclear reaction in adventitia of normal arteries (cf Fig. 1)

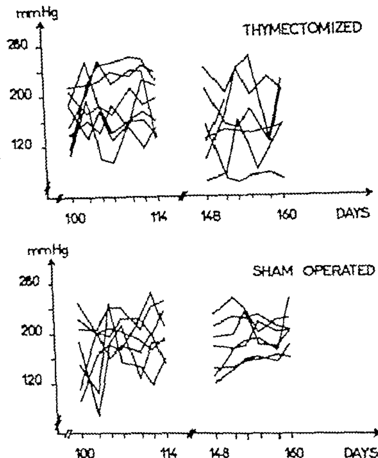
Group II 44 sham operated rats with Goldblatt clip on the left renal artery 11 males and 33 females. Litters were sham operated using the technique described above, but the incision was closed after the thymus lobes had been freed from the underlying tissue but otherwise left intact. At the age of 6 weeks the rats had their renal arteries clipped. The procedure thereafter including blood pressure measurements and histological examination, was as described for group I

Group III Control rats without Goldblatt clip 6 neonatally thymectomized 7 sham operated and 11 untouched rats When 3 months old, the thymectomized and the sham operated rats had their blood pressure recorded every second day for 4 weeks. The rats were sacrificed when 5 months old.

To investigate the effect of the thymectomy the following studies were performed.

Skin transplantation was studied by grafting squares of approximately 15 × 15 mm abdominal

Fig 2 Blood pressure curves in two periods of 14 days each for thymectomized and sham operated rats with Goldblatt clips on the renal arteries. The variation in blood pressures from day to day is that typically observed in renal hypertensive rats. No difference in the level of hypertension in the thymectomized and the sham operated rats is observed.
The abscissa: days after clipping the renal artery



skin from normal donors to the dorsal thoracic wall of the thymectomized or sham operated recipients. Grafted animals were housed singly in boxes and routinely observed several times a day for the appearance of the graft. The grafts were registered as rejected when they appeared as scabs. 15 thymectomized and 8 sham operated rats were challenged with 2 allografts ((BA × Hooded)F and (Brown Norwegian × Fischer)F) to secure at least one H 1 incompatibility and one autograft.

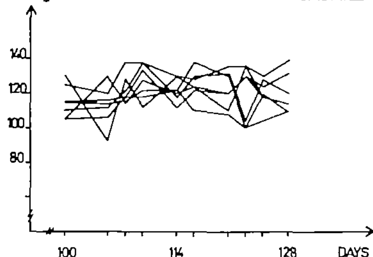
Peripheral blood lymphocyte at the age of 6 weeks, blood samples were obtained from the food pad and the number of methyl violet acetic acid stained mononuclear cells was determined using Burger Türk counting chamber 31 thymectomized and 59 sham operated rats were used

RESULTS

Eff t of skin transplantation the thymectomized rats rejected the allografts after a median time of 15 days, whereas rejection was already complete in 10 days in the control animals. 4 thymectomized animals died of wasting disease, the allografts being intact after 11 13 15 and 23 days. No difference in the rejection time of the two different allografts was observed. All autografts were accepted.

Peripheral blood lymphocytes the thymectomy was followed by lymphopenia (mean 1902 ± 873 (SD) lymphocytes per microlitre) which was significantly different from the mean in the sham operated rats (4624 ± 1639 (SD) lymphocytes per microlitre) ($p < 0.001$ Students' T test)

Autopsy of the thymectomized rats no remainder of thymus tissue was found. The two parathymic lymph nodes were usually enlarged.



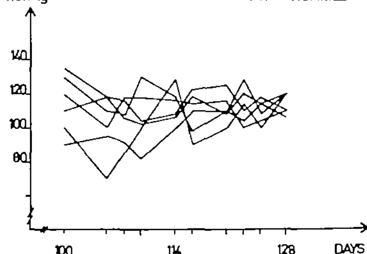


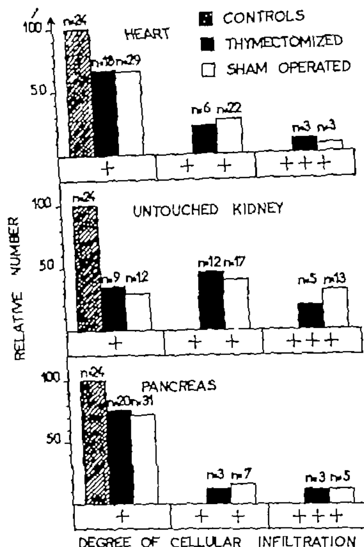
Fig 3 Blood pressure curves for 30 days obtained in a group of thymectomized (n = 6) and sham operated (n = 7) rats otherwise untreated. No difference in the level of blood pressure is observed. The abscissa: age in days.

History of Disease

Group I (thymectomized rats with Goldblatt clip) Among the 27 rats in this group one died before blood pressure determination. 18 of the remaining rats (69 per cent) became hypertensive, i.e. the mean value of the blood pressures was above 145 mm Hg. 247 blood pressures were measured in 26 rats the mean value was 170 ± 3 4 mm Hg (SEM) i.e. significantly increased as compared with the mean blood pressure in the thymectomized control rats of group III ($p<0.001$ Students T test) Typical blood pressure curves for randomly selected hypertensive thymectomized rats are shown in Fig 2. No difference was observed if blood pressures were measured for a second period 30 days after the first period. Among the 27 rats, about 22 per cent lived for 120–150 days, 56 per cent for 90–120 days and 82 per cent for 60–90 days. 13 (48 per cent) rats died spontaneously and 8 rats had to be sacrificed because they became seriously ill.

Group II (sham operated rats with Goldblatt clip) Among the 44 rats in this group, 3 died before blood pressure determination. 30 of the remaining rats became hypertensive (73 per cent) 323 blood pressures were

Fig. 4 Shows the per cent of rats with a certain degree of cellular infiltration around the arteries of the heart, untouched kidney and the pancreas. The n value is the number of rats in each column. All 3 + cellular infiltrations, nearly all 2 + cellular infiltrations and about half the 1 + cellular reactions were observed in hypertensive rats.



measured in 41 rats, the mean value was 174 ± 3.2 mm Hg (SEM) significantly higher than the mean blood pressure obtained in the sham operated control rats of group III ($p < 0.001$ Students' T test) but similar to the mean blood pressure in the group of thymectomized rats with Goldblatt clips on their renal arteries (Group I) ($p > 0.2$, Students T test) Typical blood pressure curves for hypertensive sham operated rats are shown in Fig. 2. Among the 44 rats, about 25 per cent lived for 120–150 days, 46 per cent for 90–120 days and 82 per cent for 60–90 days. 12 (27 per cent) rats died spontaneously and 22 rats had to be sacrificed because they became seriously ill.

Group III (control rats) the rats lived for 5 months. No difference between blood pressures of the thymectomized and the sham operated control rats was observed. In the thymectomized rats, 59 blood pressures were measured mean 110 ± 1.6 mm Hg (SEM) In the sham operated, 70 blood pressures were measured mean 118 ± 2.0 mm Hg (SEM) Blood pressure curves for the animals are shown in Fig. 3

Microscopic Investigations

Group I and II (Thymectomised and sham operated renal hypertensive rats) The degree of vascular disease in the heart, untouched kidney and pancreas is given in Fig 4. The figure shows that the findings were about the same in these three organs, a 3+ cellular reaction (Fig 1b) being found in only few rats, a 2+ reaction being somewhat more common, but a 1+ reaction (Fig 1a) being found in the majority of the rats. Most of the cells had morphology as lymphocytes, monocytes or macrophages. Fibroblasts and a few plasma cells were also observed. The vascular walls showed varying degrees of fibrinoid degeneration, from patchy areas of subintimal fibrinoid material up to total fibrinoid necrosis of the vascular wall (Fig 1b). Connective tissue fibrils arranged circularly around the damaged arteries were also observed. It is seen that there was no difference between the degree of cellular reaction found in the two groups in which infarcts in the heart were also found about equally often (in 37 and 28 per cent of the rats, respectively).

Group III (Thymectomised sham operated and untouched normotensive control rats) Fig 4 further shows that a 1+ cellular reaction was found in all control rats. These rats had no fibrinoid degeneration of the vessels and no infarcts of the heart.

DISCUSSION

Evidence for immunological factors in hypertensive vascular disease has accumulated. Auto-antibodies against vascular tissue have been demonstrated in rats with hypertensive vascular disease (15) and in patients with severe hypertension (5). Increased amounts of immunoglobulin in serum have been found in hypertensive patients (2, 9). If hypertensive rats are treated by methods of immunosuppression, the frequency of arteriolar damage is decreased (3). Evidence for involvement of delayed type immune reactions against substances in the vascular walls has come from experiments in which it has been possible to show that transfer of thoracic duct cells from angiotensin-II treated (8) or renal hypertensive (14) donors is followed by a changed reactivity to injections of angiotensin II, the results being that the normal recipients react with a secondary cellular response in the damaged arterioles. Inhibition of leucocyte migration by extracts from the vascular walls has been demonstrated in hypertensive patients (10). Marked hyperplasia and hypertrophy of the epithelial cells in thymus have been observed in spontaneously hypertensive rats with severe vascular lesions (13). The question concerning the pathogenic importance of these auto-immune reactions has so far remained unanswered.

It has been established that neonatally thymectomized rats have a general defect in delayed hypersensitivity reactions (1), such as skin graft rejection (4, 12) and the number of circulating lymphocytes is decreased (4, 12). This is confirmed in the present investigation. It was therefore of interest to study whether the thymus plays any role for the degree and prognosis of experimental renal Goldblatt hypertension and for the degree of vascular damage in this disease. The present study shows no such influence of the thymus. The thymectomized rats developed hypertension to a level which was not significantly different from that observed in sham thymectomized littermates, and the prognosis, based on the number of heart infarcts and spontaneous deaths, was the same in thymectomized and in sham operated rats with renal hypertension. Nor was any difference observed in the morphology or grade of the cellular infiltrations around damaged arteries in thymectomized and sham operated rats. Thus, the present investigation has failed to support the assumption that delayed type immune reactions are important in the pathogenesis of hypertensive vascular disease in renal hypertensive rats. However, since it is known that thymocytes leave the thymus and settle in the other lymphoid organs already in foetal life, the present results do not exclude that a delayed type immune reaction may have a pathogenic importance and it

would be of interest to repeat the investigation in mice with genetic lack of thymus ("nude mice"). Such experiments are in progress in our laboratory.

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DIABETOGENIC EFFECTS OF N NITROSOMETHYLUREA IN THE CHINESE HAMSTER

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Wilander E. & Gunnarsson, R. Diabetogenic effects of N-nitrosomethylurea in the Chinese hamster Acta path. microbiol. scand. Sect. A, 83 206–212 1975

To evaluate the role of the glucose residue of the diabetogenic substance streptozotocin, the effect of its aglucone derivate N-nitrosomethylurea was tested in Chinese hamsters. At a dose of 50 mg/kg body weight of N-nitrosomethylurea a triphasic blood glucose curve was recorded with an initial hyperglycaemic peak after 3 hours followed by a decrease at 6 hours. After 24 hours and during the following days the values were moderately elevated. There was a high mortality in the diabetic animals, about 60 per cent of them dying within one week. The approximate LD 50 of N-nitrosomethylurea injected intraperitoneally in non-fasting adult animals was about 125 mg/kg body weight at an observation time of 48 hours. On light microscopy degenerative changes with nuclear pyknosis were seen after 3 hours in the pancreatic islet cells, followed by cellular disintegration. Both β- a_2 and a_1-cells were obviously affected. Pretreatment with 500 mg nicotinamide/kg body weight given intraperitoneally 10 minutes before the injection of N nitrosomethylurea inhibited the hyperglycaemia and seemed to prevent the injurious effects of N-nitrosomethylurea on the islet cells. The results show that the glucose residue of the streptozotocin molecule is not necessary for the induction of diabetes in Chinese hamsters, but it seems to increase the selectivity of the toxic effects for the islet cells. This is a clear advantage in studies of experimental diabetes, especially when longer observation periods are desired.

Key words Experimental diabetes Chinese hamster N-nitrosomethylurea pancreatic islet cell degeneration hyperglycaemia.

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Streptozotocin is a substance with diabetogenic properties which were first reported by *Rakieten et al.* (1963) Beside its β-cytotoxic effect, which is well documented in laboratory rodents (*Rerup* 1970 *Rudas* 1972) it has also been shown to damage the pancreatic α_1-cells in rabbits (*Lazarus & Shapiro* 1972) and Chinese hamsters (*Wilander & Boquist* 1972) According to Herr et al. (1967) streptozotocin consists of N nitrosomethylurea linked to position C_2 of 2-deoxy-D-glucose. The N nitrosomethylurea moiety of streptozotocin, which can be regarded as the aglucone of this agent, has previously been claimed to lack diabetogenic activity (*Schein & Loftus* 1968) but has recently been shown to damage islet β-cells in mice

(*Gunnarsson et al.* 1974) Both streptozotocin and N nitrosomethylurea cause a depression of the nicotinamide adenine dinucleotide (NAD) concentration in pancreatic islet tissue. This effect is thought to be primarily involved in the β-cytotoxic action of the drug, since both the NAD depletion and the β-cell injuries can be prevented by large doses of nicotinamide (*Ho & Hashim* 1972, *Gunnarsson et al.* 1974) As similar metabolic effects were observed in the pancreatic islet tissue after injection of streptozotocin and N-nitrosomethylurea, it was decided to study the influence of the latter substance on the light microscopic morphology of the pancreatic islets and on the blood glucose concentration in the Chinese hamster an animal species in which the diabetogenic and general toxic effects of streptozotocin are well documented (*Wilander & Boquist* 1972, *Wilander* 1974a and b)

MATERIAL AND METHODS

Adult non-diabetic Chinese hamsters of both sexes were used. They were selected at random from families in which no case of overt diabetes had occurred for more than six generations. Before the experiment it was checked that the animals were free from hyperglycaemia and glucosuria. The hamsters had free access to a conventional diet for laboratory rodents and were given tap water *ad libitum*.

In one experiment, 48 non-fasting Chinese hamsters were used. N-nitrosomethylurea (Pfaltz and Bauer Flushing, New York, USA) was injected intraperitoneally in a dose of 50 100, 150 or 200 mg/kg body weight. Immediately before use, the N-nitrosomethylurea was dissolved in a citrate phosphate buffer pH 4.0 at a concentration of per cent (w/v). After an observation time of 48 hours the approximate L.D. 50 was estimated. Specimens were taken from the pancreas, liver and kidneys for light microscopic examination in animals dying spontaneously In animals still alive t o days after the injection of the drug blood glucose analyses were performed. Blood samples were taken by the orbital bleeding technique under ether anaesthesia and blood glucose was determined by a glucose-oxidase method by the aid of commercially available kit (AB Kabi, Stockholm, Sweden)

I another experiment, 59 non-fasting Chinese hamster were used. Thirty-one animals were injected intraperitoneally w th 50 mg/kg body weight of a solution of N-nitrosomethylurea prepared as described above. Sixteen control animals were given a corresponding volume of buffer solution alone. Blood samples were taken 1 3 and 6 hours after the injection and also after 1 2, 3 4 and 7 days. Tests for glucosuria were made with glucose-oxidase paper strips (Clinistix® Ames, Co., London, England) In animals dying spontaneously as well as in 17 animals killed after 0.5 1 3, 5 6 and 24 hours, specimens were taken from the pancreas, liver and kidneys for light microscopic analyses. Seven Chinese hamsters were given 500 mg/kg body weight of nicotinamide intraperitoneally 10 minutes before the injection of N nitrosomethylurea. The nicotinamide was dissolved in distilled water at a concentration of 10 per cent (w/v) immediately before use. Five controls were given 500 mg/kg body weight of nicotinamide alone. Blood samples were taken from these animals after 2 days for blood glucose analysis.

The specimens taken for light microscopic examination were fixed in Bouin's solution or in 10 per cent (v/v) formalin, dehydrated and embedded in paraffin or butoxy ethanol-glycol methacrylate (AB LAMBDA, Stockholm, Sweden) The paraffin embedded material was sectioned in 4 μ thick sections and the methacrylate embedded ma-

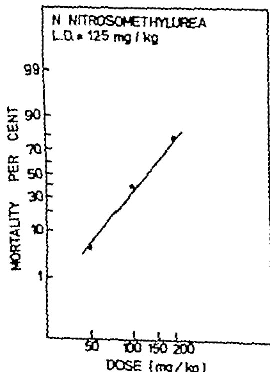


Fig 1 Dose—mortality curve after intraperitoneally administered N-nitrosomethylurea plotted on a log-probability scale.

terial in 1 μ thick sections (Sorvall JB-4 microtome). The following stains were used: Haematoxylin-eosin, van Gieson's stain, aldehyde fuchsin, and silver impregnations according to *Grimelius* (1968) and *Hellerström & Hellman* (1960).

RESULTS

LD 50 and Mortality

A linear relationship was found between the log dose of intraperitoneally injected N-nitrosomethylurea and the mortality when plotted on a log probability scale (Fig. 1). The approximate LD 50 of intraperitoneally injected N-nitrosomethylurea at an observation time of 48 hours was 125 mg/kg body weight. Fig. 2 shows the cumulated mortality after injection of 50 mg/kg body weight of N-nitrosomethylurea. The curve was sigmoid and quite steep during the 3–7 day period following the injection. Nine days after the injection, 90 per cent of the animals were dead.

Blood Glucose Concentration

After the intraperitoneal injection of 50 mg/kg body weight of N-nitrosomethylurea into 14 non fasting Chinese hamsters, a triphasic blood glucose curve was obtained (Fig. 3). There was an initial hyperglycaemic peak with a maximum after 3 hours (162 ± 11 mg/100 ml M ± SEM, $p<0.01$) followed

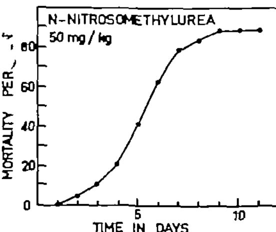


Fig. 2. Cumulated mortality in per cent in 19 Chinese hamsters injected with 50 mg N-nitrosomethylurea/kg body weight.

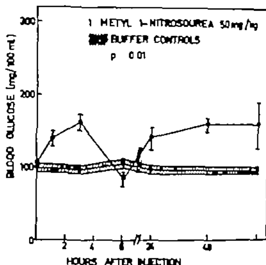


Fig. 3. Blood glucose variations (M ± SEM) in 14 N-nitrosomethylurea injected Chinese hamsters (of which 2 died 2 days after injection) and 16 controls.

by a decrease with a minimum at 6 hours (85 ± 9 mg/100 ml). After 24 hours, moderately elevated blood glucose values were again recorded (141 ± 17 mg/100 ml, $p<0.01$) and a further slight increase during the following day (159 ± 11 mg/100 ml, $p<0.01$). Two of the N-nitrosomethylurea injected animals were dead after 2 days, and another 9 after one week. No obviously decrease in the blood glucose concentration was

TABLE I. *The Effect of Nicotinamide on N-nitrosomethylurea Induced Hyperglycaemia in Chinese Hamsters. The Blood Glucose Concentrations were Determined 48 Hours after Injection*

Treatment	Blood glucose mg/100 ml M ± SEM
N-nitrosomethylurea 50 mg/kg	159 ± 11 n = 14
Nicotinamide 500 mg/kg + N-nitrosomethylurea 50 mg/kg	109 ± 10 n = 7
Nicotinamide 500 mg/kg	98 ± 6 n = 5
Buffer	96 ± 3 n = 16

$p<0.01$ in comparison with animals treated with nicotinamide + N-nitrosomethylurea.

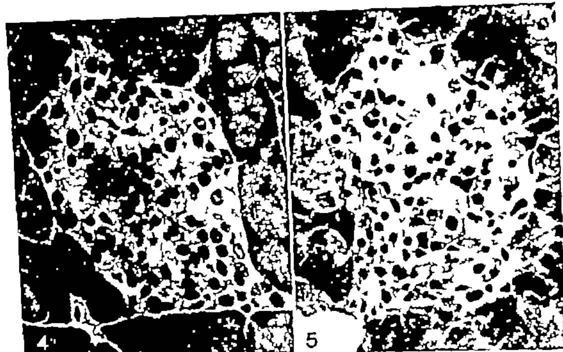


Fig 4 Pancreatic islet in a control animal one day after injection of buffer solution. Haematoxylin-eosin × 640.

Fig 5 Pancreatic islet 3 hours after injection of 50 mg N-nitrosomethylurea/kg body weight showing nuclear pyknosis and cytoplasmic vacuolization. Haematoxylin-eosin × 640.

noted up to the end of the experimental period. Several but not all of the animals dying spontaneously were hyperglycaemic. Thus, both the diabetic state and the general toxic effects of N-nitrosomethylurea seemed to cause the death of the animals. The control animals were normoglycaemic at all observation times. The experimental animals with hyperglycaemia often showed glucosuria. No glucose was observed in the urine of the control animals.

Nicotinamide

Pretreatment with 500 mg of nicotinamide/kg body weight inhibited the blood glucose elevation evoked by N-nitrosomethylurea, as seen in Table 1. The difference in blood glucose concentration between the N-nitrosomethylurea and nicotinamide + N-nitrosomethylurea injected animals was significant after 2 days ($p<0.01$). No significant difference in blood glucose concentration in animals injected with buffer alone, nicotinamide and nicotinamide + N-nitrosomethylurea was recorded ($p>0.1$).

Pancreatic Islet Light Microscopic Morphology

Thirty minutes after injection of 50 mg/kg body weight of N-nitrosomethylurea, no changes were observed in the pancreatic islets. At one hour there was slight vacuolization in a few islet cells. After three hours, nuclear pyknosis and cytoplasmic vacuolization were seen in β-cells (Fig. 5) as well as in a_1- and a-cells as revealed by silver impregnations according to *Grimelius* (1968) and *Hellerström & Hellman* (1960). No obvious degranulation occurred. During the following hours there was pronounced derangement, with karyorrhexis and fragmentation of the islet endocrine cellular structures.

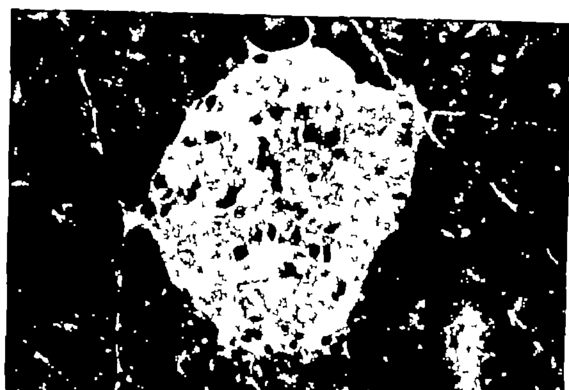


Fig 6 Pancreatic islet one day after injection of 50 mg N-nitrosomethylurea/kg body weight, showing total destruction of endocrine cellular elements in the islet tissue. Haematoxylin-eosin × 640.

The changes in the islet tissue varied to some extent in different animals, but after 24 hours this tissue contained only amorphous material and a few endothelial cells, but no endocrine cells (Fig 6) No obvious light microscopic changes occurred in islet tissue of buffer treated controls (Fig 4) Neither were there any changes in the kidneys nor in the exocrine pancreatic tissue of the experimental animals.

In the liver fatty metamorphosis was found in some cases.

DISCUSSION

In the present study injection of 50 mg of N nitrosomethylurea/kg body weight in Chinese hamsters resulted in initial hyperglycaemia at 3 hours, a blood glucose decrease at 6 hours and moderately elevated values during the following days. Streptozotocin injected Chinese hamsters also show a triphasic blood glucose curve within one day (*Wilander & Boquist* 1972) The initial blood glucose elevation after N nitrosomethylurea might be explained by an inhibition of insulin release, an effect which both streptozotocin (*Golden et al.* 1971) and N-nitrosomethylurea exhibit in isolated islets (*Gunnarsson et al.* 1974) The hypoglycaemia after 6 hours may on the other hand, be caused by insulin liberation from β-cells which at that time have reached the stage of disintegration. The marked transient hyperglycaemia observed in streptozotocin-diabetic Chinese hamsters after one day (*Wilander* 1974b) was not found in the N-nitrosomethylurea injected animals. Any simple explanation of this difference cannot be offered at present.

Since the LD 50 for N nitrosomethylurea (mol. weight 103) at an observation time of 48 hours was found to be 125 mg/kg body weight, it seems to be slightly more toxic in Chinese hamsters than streptozotocin (mol. weight 265) which has an LD 50

value of 400 mg/kg body weight (*Wilander* & *Boquist* 1972).

Similarities exist between streptozotocin and N-nitrosomethylurea in that both substances give a decrease in the NAD concentration in pancreatic islet cells. This effect can be prevented by pretreatment with nicotinamide (*Ho* & *Hashim* 1972, *Gunnarsson et al.* 1974). In Chinese hamsters (*Wilander* 1974) as in other laboratory rodents (*Schein et al.* 1967, *Schein* & *Loftus* 1968) the induction of streptozotocin diabetes can be inhibited by large doses of nicotinamide, indicating that depletion of cellular NAD may be causally related to the β-cell deterioration in this animal. This also seems to be valid for N-nitrosomethylurea diabetic Chinese hamsters in that no blood glucose elevation occurred in animals injected with 500 mg/kg body weight of nicotinamide 10 minutes before the administration of N-nitrosomethylurea.

The light microscopic changes in the pancreatic islets observed after injection of diabetogenic doses of streptozotocin and N-nitrosomethylurea seem to be similar since they occur at about the same time after injection and are seen in all types of islet cells. The results indicate that destruction of the pancreatic islet tissue, and mainly the β-cells, probably plays a major role in the development of diabetes in N-nitrosomethylurea injected Chinese hamsters. There seems, however, to be species differences in regard to the β-cytotoxic action of N-nitrosomethylurea, since β-cell destruction in mice could only be induced by very high doses of N-nitrosomethylurea (250 mg/kg body weight) (*Gunnarsson et al.* 1974)) with a high mortality rendering it difficult to study any development of permanent hyperglycaemia in these animals.

Species differences among laboratory rodents have also been found as regards the effect of other β-cytotoxic substances. Guinea pigs, for example, are reported to be resistant to the diabetogenic action of alloxan (*Hart* & *Highet* 1948) or to demand high doses of streptozotocin for induction of β-cell necrosis (*Peterson et al.* 1970, *Howell et al.* 1971).

Thus the glucose moiety of the streptozotocin molecule obviously is not necessary for induction of diabetes in Chinese hamsters, but streptozotocin seems to be a more useful diabetogenic substance for experimental studies since the general toxic effects of N-nitrosomethylurea rule out long observation periods and for instance studies of possible islet cell regeneration.

Beside the strong diabetogenic effects of N-nitrosomethylurea observed using the present doses it cannot be excluded that prolonged exposure to smaller doses of this compound could be injurious to the β-cells. This drug belongs chemically to the nitrosamines, some of which occur in low concentration in a wide variety of food products (*Crosby et al.* 1972) or can be synthesized in the mammalian stomach from various amine precursors and nitrite (*Sander* 1971). Many of the N-nitroso compounds have carcinogenic properties and the environmental nitrosamines have been considered as potentially carcinogenic in man (Ed. Lancet 1973). With regard to the present result, attention must now also be directed towards a possible diabetogenic effect of nitrosamines in man.

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AN ULTRASTRUCTURAL STUDY OF PANCREATIC ISLET CELL DESTRUCTION BY N NITROSOMETHYLUREA

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Wilander E. An ultrastructural study of pancreatic islet cell destruction by N-nitrosomethylurea. Acta path. microbiol. scand. Sect. A, 83 213–221 1975.

An ultrastructural study of the early effects of intraperitoneal injection of 50 mg/kg body weight of N-nitrosomethylurea on the pancreatic islet tissue in Chinese hamsters was performed. Thirty minutes after injection there was slight dilatation of the endoplasmic reticulum and mitochondrial swelling in a few peripheral α-cells near to capillaries and also in some β-cells. After 1 hour these changes were more pronounced. After 5 hours, distinct nuclear pyknosis was seen in endocrine islet cells of all types, together with β-cells granular irregularities, disruption of granular cores and autophagy of α-cell granules. During the following hours, marked derangement of intracellular structures, ending in obvious cellular destruction, was observed. The results indicate that N-nitrosomethylurea has a direct cytotoxic effect on the β-, α- and α-cells of the islet tissue. The similarity between the ultrastructure of the pancreatic islets after injection of N-nitrosomethylurea and streptozotocin is discussed.

Key words: Chinese hamster; N-nitrosomethylurea; pancreatic islet tissue; ultrastructure.

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In Chinese hamsters, not only the well known diabetogenic substance streptozotocin (*Wilander & Boquist* 1972) but also the N-nitrosomethylurea moiety of the streptozotocin molecule has been shown to produce overt diabetes (*Wilander & Gunnarsson* 1974) but the latter substance seems to be a less useful agent in experimental diabetic research if longer observation periods are desired as its toxic effects cause the death of most diabetic animals within one week (*Wilander & Gunnarsson* 1974). However it would seem of value to compare the functional and morphological evolution of the experimental diabetic states caused by injection of these two substances in order to elucidate their mechanisms of action. Light microscopic studies and determination of early blood glucose changes after injection of streptozotocin and of N nitrosomethylurea into Chinese hamsters have shown similar pictures. Both substances seem to affect the β-, α_2- and α_1-cells of the islet tissue. Blood glucose elevations and islet cell destruction can be inhibited by pretreatment with nicotinamide (*Wilander & Gunnarsson* 1974, *Wilander* 1974).

The purpose of the present investigation was to study the ultrastructural morphology in the pancreatic islet tissue after injection of N-nitrosomethylurea into Chinese hamsters.

MATERIAL AND METHODS

Sixteen Chinese hamsters of both sexes, five months old and weighing 25–38 gm, were used. They were selected at random from families in which no overt diabetes had been observed for more than six generations. Before the start of the experiment it was checked that the animals were free from glucosuria. They were fed a conventional diet for laboratory rodents and were given tap water *ad libitum*.

Fourteen animals were injected intraperitoneally with N-nitrosomethylurea (Pfaltz and Bauer Flushing, New York, USA) in a dose of 50 mg/kg body weight. The substance was dissolved immediately before use in a citrate phosphate buffer pH 4.0 in a concentration of 2 per cent. Two animals were given a corresponding volume of plain buffer solution intraperitoneally

In ether anaesthetized animals, an incision about 2 cm long was made in the abdominal wall and biopsies were taken from the pancreas for electron microscopic examination upon which the abdominal wall was closed in the animals which were not killed. One, two or three biopsies were taken in this way totalling 24 altogether Immediately after the last biopsy (between 0.25–6 hours after injection) the animals were killed and specimens from the rest of the pancreas were taken for light microscopic examination. They were fixed in Bouin's solution, embedded in paraffin and sectioned in 4 μ thick sections which were stained with an Gleson's strain.

Biopsies from three different animals were taken for ultrastructural analyses 0.25 0.5 1 2, 3 and 4–5,5 hours after the injection of N-nitrosomethylurea. Two biopsies from the experimental animals and 2 from normal controls were taken after 6 hours. They were fixed in 2.5 per cent glutaraldehyde in 0 1 M phosphate buffer pH 7 4 for 4 hours After rinsing in 0.1 M sucrose, the specimens were postfixed in 1 per cent osmium tetroxide for 90 minutes. All steps in the fixing process took place at +4 C. The specimens were dehydrated and embedded in Epon 812 (*Luft* 1961) After localization of islets in approximately 1 μ thick toluidine blue stained sections, ultra thin sections were prepared by an LKB Ultrotome The sections were mounted on Formvar-coated copper grids, contrasted with uranyl acetate (*Watson* 1958) and lead citrat (*Reynolds* 1963) and examined in a Zeiss electron microscope EM 9 at 60 kV

RESULTS

Light Microscopic Morphology

Specimens from the pancreas taken at sacrifice from the animals injected with N-nitrosomethylurea showed islet cell degeneration and necrosis just as previously described (*Hellander & Gunnarsson* 1974) In the buffer controls, no changes were noted in the islet tissue

Electron Microscopic Morphology

Fifteen minutes after injection of N-nitrosomethylurea all islet cells appeared normal The first alterations were seen after 30 minutes, consisting in slight mitochondrial swelling and dilatation of the endoplasmic reticulum in a few α_2-cells (Fig. 1) near to capillaries as well as in some β-cells (Fig. 2)

At 1 hour the mitochondrial swelling and intracytoplasmic vacuolization were more pronounced in the α_2-cells and β-cells. β-cells containing intracytoplasmic dense bodies (Fig 3) and α_2-cells with secretory granular cores incorporated in lysosomes (granulophagy) (Fig 4) were also seen at this point, as well as granular material in the endoplasmic reticulum of some α_2-cells.

Three hours after injection of N nitrosomethylurea, distinct condensation of nuclear chromatin was observed in β- α_2- and α_1-cells (Figs. 5 and 6) At the same time and during the following hours, the β-cell granule limiting membranes sometimes ruptured and the secretory granular cores showed marked irregularities and even disappearance leaving an electron lucent space (Fig 7) No degranulation occurred In the α_2-cells, dilatation and even rupture of the granule limiting membrane occurred (Fig 8)

At the end of the observation period, the degenerative changes were more pronounced, including nuclear karyorrhexis and marked disintegration of intracytoplasmic structures, resulting in cellular destruction. Because of

Fig 1 An α_2-cell 30 minutes after injection of N-nitrosomethylurea, presenting mitochondrial swelling and dilatation of the endoplasmic reticulum × 17 700.

Fig 2 β-cells 30 minutes after injection of N-nitrosomethylurea, presenting swollen mitochondria and intracytoplasmic vacuolization × 17 700




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the pronounced changes it was often difficult at that time to identify the different types of cells in the islets.

During the later part of the experimental period, macrophages with engulfed granules from disintegrated endocrine islet cells were seen. This granulophagy differed from that observed earlier in α_2-cells in that the granule limiting membranes were sometimes preserved after incorporation into the lysosomes (Fig. 9).

There were only slight variations in the ultrastructural picture of the islet tissue in different animals studied at the same observation times. No obvious alterations in the exocrine pancreatic tissue of N-nitrosomethylurea treated Chinese hamsters were found. In endocrine pancreatic tissue of buffer injected animals, no alterations including dense bodies in the cytoplasm of β-cells were found.

The repeated biopsies of the pancreas in some animals did not seem to affect the islet cell morphology since the same picture was observed in pancreatic glands from which only one biopsy was taken.

DISCUSSION

Other reports on the ultrastructural morphology of the pancreatic islet tissue after injection of N-nitrosomethylurea are apparently not available. In streptozotocin injected rats *Arison et al.* (1967) found β-cell degranulation without obvious necrosis and *Lazarus & Shapiro* (1972) observed dilated endoplasmic reticulum and swollen mitochondria in rabbit β-cells and α_2-cells after 3 hours, followed later by necrosis of these cells. On the ultrastructural level, streptozotocin causes a selective destruction of the islet β-cells in guinea pigs, leaving the α_1- and α_2-cells completely intact (*Howell & Whitfield* 1972). The first ultrastructural changes in the pancreatic islets of Chinese hamsters after injection of streptozotocin occur after about 30 minutes and are in the form of aggregates of secretory granules in some β-cells, as observed in Chinese hamsters with spontaneous hereditary diabetes (*Boquist* 1969). At 1½ hours, cytoplasmic vacuolization of varying degrees and mitochondrial swelling are seen in the streptozotocin injected animals. Pyknosis in β-cells occurs after 3 hours in these animals together with disruption of granule limiting membranes and irregularities or disappearance of granular cores. Degenerative changes also take place in α_2-cells and lysosomes with incorporated granules are seen both in β- and α_2-cells (*Wilander & Boquist* 1972). The degenerative changes in the streptozotocin injected Chinese hamster greatly resemble those observed after injection of N-nitrosomethylurea in the same animal except for autophagy of granules in the β-cells in the former. The space of time for the destruction of islet endocrine cells by streptozotocin and N-nitrosomethylurea seems to be about the same.

Similarities in the ultrastructural morphological changes in islet tissue caused by strep-

Fig. 3. Dense bodies at least partly surrounded by limiting membranes in β-cell cytoplasm 1 hour after injection of N-nitrosomethylurea. × 17 700.

Fig. 4. Peripherally located α_2-cell one hour after injection of N-nitrosomethylurea with typical secretory granules some of which have lost their limiting membranes and are incorporated in lysosomes. × 17 700.

Fig. 5. Part of pancreatic islet 3 hours after injection of N-nitrosomethylurea, showing nuclear pyknosis in a β-cell (right), an α_2-cell (middle) and an α_1-cell (left). Intracytoplasmic vacuolization of the β- and α-cells is also seen. × 9 100.

Fig. 6. Part of an α-cell 3 hours after injection of N-nitrosomethylurea, presenting typical secretory granules, slight mitochondrial swelling and condensation of nuclear chromatin. × 17 700.

Fig. 7. Pancreatic islet β-cell 3 hours after injection of N-nitrosomethylurea. Nuclear pyknosis, irregularities or disappearance of granular cores, disruption of granular membranes and mitochondrial swelling are seen. × 17 700.

Fig. 8. Peripherally located α_2-cell 3 ½ hours after injection of N-nitrosomethylurea. Nuclear pyknosis and secretory granules with dilated or disrupted limiting membranes are seen. × 17 700.

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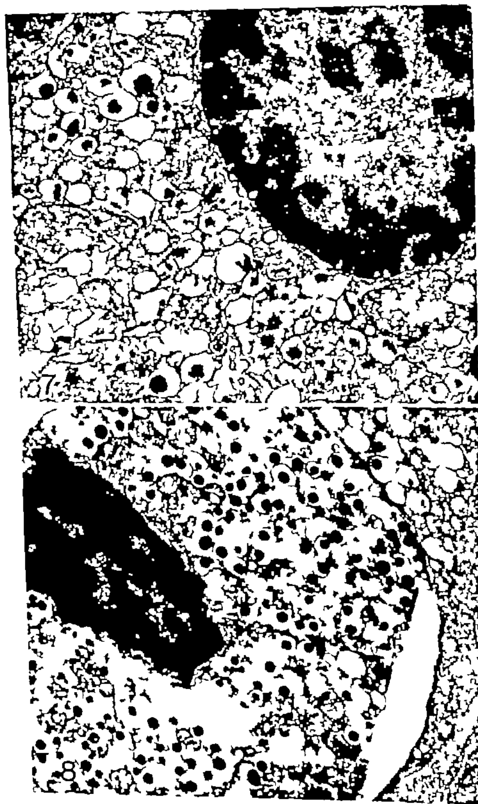

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Fig 9 Macrophage in pancreatic islet 5 hours after injection of N-nitrosomethylurea with phagocytosis of endocrine granular material × 17 700

tozotocin and N nitrosomethylurea may indicate that their mode of action in this tissue is identical. However the ultrastructural alterations are non-specific and are also found in pancreatic islet β-cells in alloxan treated Chinese hamsters, although they appear there after only 5 minutes. The difference between alloxan on the one hand and streptozotocin and N-nitrosomethylurea, on the other is obvious, however since alloxan only causes slight changes in α_2- and α_1-cells which have been explained as secondary to the extensive β-cell damage (*Boquist* 1968) The present study indicates that N nitrosomethylurea has a direct cytotoxic effect on the islet tissue β α and α -cells in Chinese hamsters, as is also probable in the case of streptozotocin.

It may be of interest to note that the specificity of streptozotocin for β-cells varies in different animal species (*Wilander* & *Boquist* 1972 *Lazarus* & *Shapiro* 1972 *Howell* & *Whitfield* 1972) The fact that both streptozotocin and N nitrosomethylurea destroy all types of endocrine cells of the islet tissue in Chinese hamsters may favour the hypothesis advanced by *Pearse et al.* (1973) namely that all these cells arise from common precursor cells of neuro-ectodermal origin. However whether or not the cytotoxic action of streptozotocin and N nitrosomethylurea on the α-cells is a result of a lowering of the cellular nicotinamide adenine dinucleotide levels, as is probable in the β-cells (*Gunnarsson et al.* 1974) is not known

Autogranulophagy which has been interpreted as lysosomal digestion of unneeded secretory granules (*Smith* & *Farquhar* 1966) has been observed in pancreatic islet α_2-cells in spontaneously diabetic animals (*Orci et al.* 1968, *Orci et al* 1970) in streptozotocin diabetic rats (*Orci et al.* 1969 In Chinese ham-

sters (*Wilander* & *Boquist* 1972) and also, but rarely in normal hamsters (*Orci et al* 1970). Further evidence in support of the assumption that autogranulophagy represents "auto digestion" of unneeded secretory products is given by *Creutzfeldt et al.* (1969) who found autophagy of β granules after inhibition of insulin release by diazoxide in rats. In the present study granules incorporated into lysosomes were seen in α_2-cells after injection of N-nitrosomethylurea. *Golden et al.* (1971) and *Gunnarsson et al.* (1974) have shown that streptozotocin exerts an inhibitory effect on insulin release in *vitro*. It is possible that N-nitrosomethylurea and streptozotocin have a similar effect on the glucagon release in α_2-cells.

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CYTOKINETIC VARIATIONS DURING AGEING AND REGENERATIVE GROWTH IN THE JB-1 ASCITES TUMOUR STUDIED BY IMPULSE CYTOPHOTOMETRY

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Dombernowsky, P. & Bichel, P. Cytokinetic variations during ageing and regenerative growth in the JB-1 ascites tumour studied by impulse cytophotometry. Acta path. microbiol. scand. Sect. A, 83: 222-228, 1975.

The variation in the DNA distributions in the murine JB-1 ascites tumour was investigated by means of impulse cytophotometry (ICP) on days 2, 4, 7, 10 and 14 after i.p. inoculation of 2.5×10^{6} tumour cells and on days 1, 2, 3 and 4 after aspiration of the main part of 10-day plateau tumours. The study showed that a considerable fraction of cells with a DNA content of about 2 and 4 n ($p<0.001$) accumulated from day 2 to 10. During the same period the fraction of cells in S decreased significantly ($p<0.001$). One and two days after aspiration, a rapid rearrangement in the distribution pattern occurred, leading to a predominance of cells with S DNA content, while the fraction of cells around 2 n and 4 n decreased. Three and four days after aspiration the cells again accumulated around the 2 and 4 n values while the fraction of cells with S DNA content decreased. The ICP results were in agreement with our data obtained by autoradiography and Feulgen microspectrophotometry, and ICP is recommended as a rapid and statistically precise method in cytokinetic analysis.

Key words: JB-1 ascites tumour; cytokinetics; impulse cytophotometry.

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It has been shown in many studies that the growth rate of ascites tumours decreases with increasing tumour mass and time after inoculation (6, 8, 10, 15, 16, 19).

In previous investigations of the JB-1 and the L1210 ascites tumours we observed that the decelerating growth was accompanied by an accumulation of resting cells, an increase in the cell loss and, in general, a prolongation of the mean cell cycle time (2, 6, 7, 8, 13). In the JB-1 tumour the accumulation of resting cells first with G_1 DNA content (Q_1 cells) and later also with G_2 DNA content (Q_2 cells) was demonstrated by the combination of autoradiography, Colcemid block and determination of single-cell Feulgen DNA content by a microspectrophotometric method. It was also observed that Q_1 and Q_2 cells were rapidly triggered into the cell cycle after retransplantation (2), in the recurrent phase of growth (7) and after cytostatic treatment (5).

In our previous investigations we used

Feulgen microspectrophotometry for the determination of single-cell DNA content, a technique which is very time consuming (12). However, in the last few years instrumentation has been available for rapid determination of single-cell DNA content from many cells (9, 12, 21). Using flow microfluorometry or impulse cytophotometry (ICP) changes in the DNA distributions after cytostatic treatment and irradiation have been analysed (11, 14, 20).

ICP is working on principles not previously used in our cytokinetic studies of ascites tumours. The purpose of this study was therefore to re-investigate the variations in the DNA distributions in the JB-1 ascites tumour with increasing tumour age and in the recurrent growth and to compare these results with those obtained by our autoradiographic and microspectrophotometric analyses.

MATERIALS AND METHODS

The JB-1 ascites tumour used in the present study is a hypotetraploid transplantable plasmacytoma maintained syngeneically in inbred AKR/Aa mice by intraperitoneal (i.p.) injection of 5×10^6 tumour cells every ninth or tenth day. The origin of the tumour has been described previously (1).

The mice used in the study were inoculated with 2.5×10^6 tumour cells on day 0 and, in the studies of the age variations, about 0.1 ml ascites fluid was aspirated on days 2, 4, 7, 10 and 14 for ICP analysis. In the studies of the recurrent growth as much as possible of 10-day plateau JB-1 ascites tumours was withdrawn by aspiration in the flank using a 21 gauge needle and from day 1 to 4 after aspiration about 0.1 ml ascites fluid was withdrawn for ICP analysis.

The tumour cells to be measured in the ICP were washed twice with 0.9 per cent saline, fixed in 96 per cent ethanol during vigorous magnetic stirring to ensure a single-cell suspension, and treated with an aqueous solution of ribonuclease (0.25 mg/ml, BDH, obtained from bovine pancreas) for 1 hour at 37 °C. The cells were then stained with ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, BDH, 10 µg/ml in Tris-HCl buffer pH 7.5). Ethidium bromide is a fluorochrome which stains doublestranded nucleic acid with high selectivity (17). After filtration of the cell suspension through a 100 µ filter the relative DNA content of the individual cells in suspension was measured fluorometrically using an ICP 11 impulse cytophotometer (Phywe AG, Göttingen, West Germany) constructed according to the following principles as described by Göhde & Dittrich (12): The suspensions of cells in dye solution are sucked through a 80 µm aperture in front of a fluorescence microscope combined with a photomultiplier. Light from a mercury lamp excites the ethidium bromide and causes a flash fluorescence proportional to the amount of dye present in the cell. The pulses are sorted and stored in a multichannel analyser and presented automatically as a plot of the number of measured cells versus the relative fluorescence intensity (or relative DNA content) of the individual cells on a chart recorder.

From 5.5×10^4 to 16.5×10 cells in each tumour were measured at a maximum rate of 1000 cells/sec.

The proportions of cells with G_1, S and G DNA content were estimated from the histograms by planimetry (Fig. 1). In the histograms the first large peak represents normal host cells, the second represents JB-1 ascites tumour cells with G_1 DNA content ($G + Q$ cells) and the peak at twice the modal number represents cells with G DNA content ($G + Q_2$ + cells in mitosis). Cells in the S-phase with a varying degree of DNA synthesis completed are distributed between the last two peaks. The cells in S were estimated as the quadrangle between two vertical lines through the G_1 and the G peaks and a horizontal line through the trough between G and G_2 (18). If a vertical line is drawn halfway between the G and the G_2 values, the area to the left of this line represents $G + \frac{S}{2}$ cells, and that to the right $G + \frac{S}{2}$ cells, from which the amount of cells with G and G content can be calculated.

Student's t test was used in the statistical analysis.

RESULTS

Fig. 1 shows a typical DNA histogram for a JB-1 ascites tumour 10 days after inoculation. Three peaks are seen in the histogram: the first represents normal host cells such as lymphocytes, granulocytes and macrophages (i.e. cells with normal mouse DNA content), the second and third peaks represent hypotetraploid tumour cells with G and G values, respectively, while S-phase cells give relative fluorescence values between the G_1 and G maxima.

In Fig. 2 are shown DNA histograms for tumours 2, 4, 7, 10 and 14 days after inoculation and in the recurrent growth on days 1 to 4 after aspiration of 10-day plateau tu-

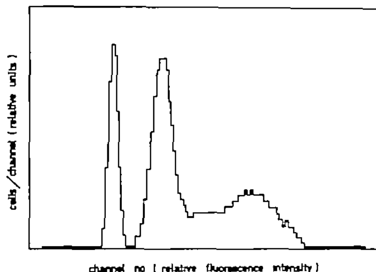


Fig 1 Impulse cytophotometric determination of DNA content of a 10-day JB-1 tumour Ordinate cells/channel (relative units) Abscissa channel number (relative fluorescence intensity) Peak 1, normal host cells Peak 2, tumour cells with G_1 DNA content Peak 3 tumour cells with G_2 DNA content.

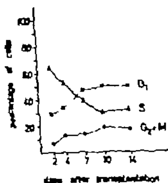
2-day tumour | 4-day tumour | 7 day tumour | 10-day tumour | 14-day tumour

day 1 after aspiration | day 2 after aspiration | day 3 after aspiration | day 4 after aspiration

cells/channel (relative units)

channel no. (relative fluorescence intensity)

Fig 2 DNA histograms of JB-1 ascites tumour cells 2, 4 7 10 and 14 days after i p inoculation of 2.5×10^4 JB-1 cells, and 1 2 3 and 4 days after aspiration of 10-day JB-1 ascites tumours. Ordinate cells/channel (relative units) Abscissa channel number (relative fluorescence intensity) Each diagram represents a measurement of 10 single tumour cells; the peak representing normal cells is omitted.



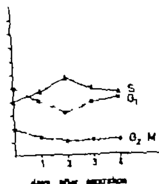


Fig 3 Percentages of cells with G_1, S and G_2 DNA content as a function of time after i p. inoculation of 2.5×10^6 JB-1 tumour cells on day 0 (left) and days 1 2, 3 and 4 after aspiration of 10-day plateau JB-1 ascites tumours, (right) Each point represents values from 5 to 13 mice.

TABLE 1 *Results of Impulse Cytophotometric Determination of Single-Cell DNA Content in JB-1 Tumour Cells*

	No. of tumours	*Percentage distribution* G + Q_1	S	G_2 + Q_2 + M
2-day tumour	5	29 4 ± 1.5‡	63 7 ± 1.6	6.9 ± 0 4
4-day tumour	7	34 0 ± 1.5	53.0 ± 1.5	13 0 ± 0.9
7-day tumour	10	47.3 ± 1 4	38.8 ± 1.0	13.9 ± 0.9
10-day tumour	10	50.0 ± 0.7	30.7 ± 1 1	19.3 ± 0.9
14-day tumour	6	49.4 ± 1 7	33 7 ± 1.6	16.9 ± 1.2
Day 1 after aspiration	10	39.5 ± 3.2	47.2 ± 2 1	13.3 ± 1.9
Day 2 after aspiration	10	31.9 ± 1.3	57 7 ± 1.3	10.4 ± 1.2
Day 3 after aspiration	10	39.2 ± 1 7	49.8 ± 1.3	11.0 ± 0.7
Day 4 after aspiration	13	42.1 ± 0.7	46.9 ± 1.5	11.0 ± 1 1

Cells in mitosis are included in the fraction of cells with G DNA content.
‡ Represents standard error of the mean.
The tumours were investigated on days 2, 4 7 10 and 14 after an i p inoculum of 2.5×10^6 cells, and on days 1 2 3 and 4 after aspiration of the main part of 10-day plateau JB-1 tumours.

mours. The peak representing normal cells is omitted in these curves.

The calculated fractions of cells with G_1 S and G DNA content with increasing tumour age and in the recurrent growth are shown in Table 1 and Fig 3

Table 1 and Fig 3 show that the percentage of cells in S decreased significantly ($p<0.001$) from day 2 to 10, viz. from 63 7 to 30.7 per cent, while no significant variation was observed from day 10 to 14 ($p>0$ 10) The reduction in the fraction of cells with S DNA content from day 2 to 10 was paralleled by a significant increase ($p<0.001$) *in* the percentage of cells with G DNA content from 29 4 to 50.0 per cent and a significant increase ($p<0.001$) from 6.9 to 19.3 per cent in the fraction of cells with G_2 DNA content.

Aspiration of as much as possible of 10-day plateau tumours caused one and two days later significant increases in the percentage of cells with S DNA content, from 30 7 per cent in the 10-day tumour to 47.2 and 57 7 per cent ($p<0.001$) respectively In the same period, the fraction of cells with G DNA content decreased from 50.0 to 31.9 per cent ($p<0.001$) and the fraction of cells with G DNA content decreased from 19.3 to 10 4 per cent ($p<0.001$)

On days 3 and 4 after aspiration, the fraction of cells in S again decreased to 49.8 and 46.9 per cent ($p<0.001$) respectively and at the same time the percentage of cells

with G_1 DNA content increased to 39.2 and 42.1 per cent as compared with 31.9 per cent on day 2 after aspiration ($p<0.005$ and 0.001 respectively). The percentage of cells with G DNA content remained on a level of about 10–11 per cent ($p>0.1$).

DISCUSSION

Our study showed that, with increasing age of the JB-1 ascites tumour a considerable fraction of cells accumulated around the 2 and 4 n DNA values. When the main part of the plateau tumour was removed, a rapid rearrangement in the distribution pattern occurred, leading to a predominance of cells with S DNA content, while the cells again accumulated around the 2 and 4 n values on days 3 and 4 after aspiration. These impulse cytophotometric results were in agreement with previous data obtained by autoradiography and Feulgen microspectrophotometry.

In a previous investigation of the JB-1 tumour we observed an increase in the doubling time from 22.8 to 240 hr from day 4 to 10 after transplantation, a prolongation of the mean cell cycle time from 14 to 41 hr and a decrease in the growth fraction from 76 to 44 per cent (6). It was also observed that the decrease in the growth fraction was due to a transition of cycling cells with G and G_2 DNA content into non-cycling stages (Q_1 and Q_2) together with a decrease in the fraction of cells in S. At 24 hr after aspiration of the main part of the ascites tumour (approximately 80 per cent) we observed a decrease in the doubling time from 240 to 73 hr and an increase in the growth fraction from 44 to 72 per cent as compared with the plateau tumour (7). The increase in the growth fraction was due to an increase in the fraction of cells in S parallel to a decrease in the percentage of cells with G DNA content, while the fraction of cells with G DNA content remained unchanged.

Comparison of the fractions of cells with G_1, S and G DNA content in the case of the 10-day tumour obtained by ICP and autoradiography combined with cytophotometry (6) showed values of 50.0, 19.3 and 30.7 per cent, 47.8, 18.0 and 34.? per cent, respectively. Comparison of the two methods on day 1 after aspiration also revealed a reasonable agreement. However the fraction of cells in S in the 4-, 7- and 10-day tumour determined by ICP were constantly less than those determined by autoradiography. This can be explained from the grain limit of 3 or more grains used in the evaluation of the autoradiographs. This grain limit is low and only slightly above the mean background (average 1–2 grains). Consequently the experimental error in the autoradiographs is an overestimation of the percentage of labelled cells and an underestimation of the percentage of unlabelled cells.

The results obtained in this study were based on the ICP technique, and it might be reasonable to discuss some of the factors which are able to introduce errors into the results.

ICP measurements require single cells in suspension and care must be taken to avoid selective loss of certain classes of cells during sample processing (9). If the cells are clumping before or during the preparation procedures, cells would be seen at the 6 and 8 n quantities, and such peaks did not occur.

Occasionally two cells are measured at the same time. This coincidence rate is about 2 per cent at a measuring rate of 1000 cells/sec (9). The coincidence gives a fraction of cells with G DNA that is too high. This probably was the case in some situations, e.g. in the 4-day tumour in which the G + M fraction was 8.9 per cent as determined by autoradiography and cytophotometry as against 13.0 per cent obtained with ICP.

Another possible error in the DNA measurement is contamination with normal cells such as lymphocytes, granulocytes and macrophages which in advanced ascites tumours may represent from about 10 to 50 per cent of the total population (15). The JB-1 ascites tumour is hypotetraploid (69–73 chromosomes, Bichel, unpublished observation) and without significant deviations in chromo-

some number As far as our tumour is concerned, ICP allows an excellent distinction between normal host cells and tumour cells, which means that the normal cells show a well-defined peak at about 20 arbitrary units, as compared with about 36 arbitrary units in the case of tumour cells with G_1 DNA content.

ICP gives only the fractions of cells with G_1, S and G_2 DNA content and does not permit a differentiation between cycling and non-cycling cells. This differentiation is possible only when much more complicated and time consuming methods are used, such as per cent labelled mitosis analyses and continuous labelling experiments combined with microspectrophotometric determination of single-cell DNA content (7 13) However such experiments need durations of about 2 days, and in this period, the cytokinetic parameters of dynamic tumour systems continually change whereas ICP gives a "snapshot" of the cytokinetics.

The mathematical problems involved in the ICP measurements are not yet completely solved. Computer methods for the calculation of the different fractions from ICP data have been reported (4 18) These methods are based on the same principles as those used in our work (18) or on more complicated mathematical methods (4) It applies to both methods that the ICP data show a good fit to the values obtained from growth curves and per cent labelled mitosis analyses.

Despite the drawbacks mentioned, the use of ICP for the determination of single-cell DNA content permits a statistical precision which cannot be obtained by microspectrophotometry This makes such measurements particularly valuable for the demonstration of small and rapid fluctuations in the cell kinetics. Therefore we consider this technique to be a very useful supplementary method in cytokinetic analysis

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SPREAD OF TUBERCULOSIS FROM OBSTRUCTED AND NON OBSTRUCTED UPPER URINARY TRACT

An Experimental Study in Male Guinea Pigs

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Winblad, B. & Ducheck, B. Spread of tuberculosis from obstructed and non-obstructed upper urinary tract An experimental study in male guinea pigs. Acta path. microbiol. scand. Sect. A, 83 229-236, 1975.

As part of an experimental study of the spread of urogenital tuberculosis, male guinea pigs were inoculated with tubercle bacilli ($H_{37}Rv$) in the upper part of the ureter Half of the animals were prepared by ligature of the ureter below the injection site prior to inoculation. Spread of the infection to the lower urinary tract and the genitalia occurred whether the ureter was intact or ligated. It is concluded that lymphatic spread is possible and seems to be more common than urinary (canalicular) spread.

Key words Uro-genital tuberculosis experimental tuberculosis spread tuberculosis lymphatic spread experimental model guinea pig.

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From most clinical and experimental studies it follows that in the male the primary manifestation of urinary tract tuberculosis is in the kidney It has also been shown that the primary kidney focus of infection arises through haematogenous spread. The spread of tuberculosis in the urinary tract and to the genitalia has been considered to occur via the urinary (canalicular) route (*Hansen* 1907 *Joller* 1949 *Ljunggren* 1959 *Mazurek* 1963) but the additional possibility of lymphatic spread has been implied in some publications according to which tuberculosis in the ureteral wall and the surrounding lymphatics has been observed (*Bauereisen* 1911 *Ljunggren* 1959) More disputable is the question whether an ascending infection from the urinary passages to the kidneys occurs (*Albarran* 1891 *Baumgarten & Kraemer* 1905 *Wildbolz* 1909)

Our previous investigations have shown that lymphatic spread of injected tubercle bacilli in guinea pigs occurs between the urinary bladder and genitalia (*Duchek & Winblad* 1973) and also between the pelvic and scrotal genital organs (*Duchek & Winblad* 1973) This paper presents a study of the spread of tuberculous infection after intra ureteral injection utilizing a modification of the experimental model previously applied (*Duchek & Winblad* 1973)

MATERIAL AND METHODS

Experimental Animals

Twenty-six sexually mature male guinea pigs, bodyweight approx. 300 g were inoculated with tubercle bacilli into the lumen of the left ureter



Fig 1 Injection into left ureter (U) after ligation of ureter distal to injection site and separation from peri-ureteral tissue (arrow) Another ligature is placed proximally to injection site around ureter and needle and ligated during withdrawal of the needle Left kidney (LK) visible at right top.

Half of the animals were prepared by ligature of the left ureter below the injection site. The animal were individually caged and had free access to food and water

Inoculum A 14-day-old culture of tubercle bacilli, H R diluted with physiological saline to a concentration of 0 1 mg per ml containing approximately 50 000 colony forming units per ml, was used as inoculum.

Operative Procedure

The animals were randomly divided into two equal groups. Mebumal sodium 2.5 mg per 100 g body weight was administered intraperitoneally supplementary ether anaesthesia was used on occasion. The abdomen was opened by a midline incision. In one group (I) the left ureter was exposed and 0.05 ml of inoculum was injected in retrograde direction into the proximal part of the ureter from a disposable tuberculin syringe In the other group (II) the animals were inoculated with the same dose and in the same way as in group I but the ureter was first ligated just distal to the injection site after careful separation from its surrounding tissue under a dissecting microscope. Before the needle was withdrawn, another ligature was placed proximally to the injection site around the ureter and the needle and tied during withdrawal of the needle (Fig 1) In both groups, a cotton-tipped applicator stick soaked with a strongly bactericidal plastic material, well tolerated by tissues (Nobecutan® Bofors) was applied to the injection site This was held in place for about a minute after completion of the injection. The abdominal wall was closed in two layers. The animals were killed after 4 weeks.

Identification of Tuberculous Infection

The tuberculous infection was evaluated by macroscopic inspection and microscopic examination of tissue specimens fixed in neutral formalin and embedded in paraffin Histological sections were stained with haematoxylin-eosin and, for demonstration of tubercle bacilli, with a fluorescent stain (auramine-rhodamine) (*Winblad & Duchek* 1973)

The following organs were examined left ureter below (LUD) and above (LUP) the ligature, kidneys, urinary bladder prostate, seminal vesicles, epididymes, testes, spleen, iliac para-aortic and renal lymph nodes. The demonstration of tubercle bacilli in combination with characteristic morphological tissue changes formed the criteria for characterizing an organ as tuberculous. Urine aspirated by direct puncture of the bladder at autopsy and blood taken from the heart were cultured on Löwenstein-Jensen's medium

RESULTS

The results are shown in Table 1 and 2 and reviewed in Figures 2 and 3. In group I (without ligature of the left ureter) three animals died at 3 6, and 8 days after operative procedure. In group II (with ligature of the left ureter) five animals died at 3 6, 8, 8, and 20 days after operation. As regards the animals which died after 3 8 days, the cause of death was intestinal volvulus, respectively ileus, caused by adhesions. The animal which died after 20 days showed also an enormous hydronephrosis of the left side that took up almost the whole abdominal cavity

In group I there was a slight weight increase in most of the animals, while weights

TABLE 1 *Spread of T bereulosis in the Male Uro-Genital System. Inoculation into the Left Intact Ureter (Group I)*

Animal	LE	LSV	BP	LUD	LUP	LK	RSV	RE	RK	U	B
1	+	+	+	+	+	−	−	+	−	+	−
2	−	−	+	+	+	+	+	+	−	+	−
3	−	−	−		+	−	−	−	−	−	−
4	−	−	−	+	+	−	+	−	−	−	−
5	−	+	−	+	−	−	+	−	−	−	−
6	−	−		+	+	−	−	−	−	+	−
7	+	−	+	+	+	+	−	−	−	+	−
8	+	−	+	+	+	+	−	−	−	−	−
9	−	−	−		+	+	−	−	−	+	−
10	−	−	−	+	+	+	−	−	−	+	−

TABLE 2. *Spread of Tuberculosis in the Male Uro-Genital System. Inoculation into the Left U eter after Ligation of the U eter (Group II)*

Animal	LE	LSV	BP	LUD	LUP	LK	RSV	RF	RK	U	B
11	+	+	+	+	+	+	−	−	−	−	−
12	−	+	−	+	+	+	−	−	−	−	−
13	+	−	+	+	+	+	−	+	−	−	−
14	−	−	−	−	+	−	−	−	−	+	−
15	−	−	−	+	+	+	−	−	−		−
16	−		−	+	+	+		+	−	−	−
17	−	−	−	−	+	+	−	−	−	−	−
18	−	−	−	−	+	+	−	−	−	+	

□ Indicates the site of inoculation.
\+ Indicates tuberculous inflammation.
− Indicates absence of tuberculous changes.
LE Left epididymis.
LSV Left seminal vesicle.
BP Bladder-prostate.
LUD Left reter distal.
LUP Left ureter proximal.
LK Left kidney.
RSV Right seminal esicle.
RE Right epididymis.
RK Right kidney.
U Löwenstein-Jensen culture on urine.
B Löwenstein-Jensen culture of blood.

of those in group II decreased, on an average, by 35 grams. There was no peritoneal tuberculosis in any animal from either group I or group II and no signs of miliary tuberculosis were seen.

In the group of animals with ligated ureter (group II animals 11–18) all the animals had a left-sided uretero-hydronephrosis with a thick, milky content which partly adhered to the mucous membrane of the kidney pelvis, and the tip of the papilla. The ureter exhibited a thickened wall and was dilated down to the ligature. Even in the group of animals without ligature of the ureter (group I animals 1–10) uretero-hydronephrosis extending down to the injection site occurred in three of the animals (7 9 10). However urine passage was observed in animal 7 this animal having only a partly occluded ureter while ureteral occlusion in the other two animals was complete.

In all animals, both those without and those with ureteral ligatures, greyish-white longitudinal streaks were observed on the outer wall of the ureter both proximal and distal to the ligatures and extending out to the adjacent, enlarged para-aortic lymph nodes.

The animals of both groups, except one (animal 5) exhibited tuberculous changes in



Fig 1 Injection into left ureter (U) after ligation of ureter distal to *injection site* and separation from peri-ureteral tissue *(arrow)* Another ligature is placed proximally to injection site around ureter and needle and ligated during withdrawal of the needle Left kidney (LK) visible at right top.

Half of the animals were prepared by ligature of the left ureter below the injection site. The animals were individually caged and had free access to food and water

Inoculum A 14-day-old culture of tubercle bacilli H_3 R diluted with physiological saline to a concentration of 0 1 mg per ml containing approximately 50 000 colony forming units per ml was used as inoculum.

Operative Procedure

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4	−	−	−	+	+	−	+	−	−	−	−
5	−	+	−	+	−	−	+	−	−	−	−
6	−	−	−	+	+	−	−	−	−	−	−
7	+		+	+	+	+	−	−	−	+	−
8	+	−	+	+	+	+	−	−	−	+	−
9	−	−	−	−	+	+	−	−	−	−	−
10	−	−	−	+	+	+	−	−	−	+	−

TABLE 2. *Spread of Tuberculosis in the Male Uro-Genital System. Inoculation into the Left Ureter after Ligation of the Ureter (Group II)*

Animal	LE	LSV	BP	LUD	LUP	LK	RSV	RE	RK	U	B
11	+	+	+	+	+	+	−	−	−	−	−
12	−	+	−	+	+	+	−	−	−	−	−
13	+	−	+	+	+	+	−	+	−	−	−
14	−	−	−	−	+		−	−	−	+	−
15	−	−	−	+	+	+		−	−	−	−
16	−	−	−	+	+	+	−	+	−	−	−
17	−	−	−	−	+	+		−	−	−	−
18	−	−	−	−	+	+	−	−	−	+	−

▢	Indicates the site of inoculation.	LUP	Left ureter proximal.
+	Indicates tuberculous inflammation	LK	Left kidney
−	Indicates absence of tuberculous changes	RSV	Right seminal vesicle.
LE	Left epididymis.	RE	Right epididymis.
LSV	Left seminal vesicle.	RK	Right kidney
BP	Bladder-prostate.	U	Löwenstein-Jensen culture on urine.
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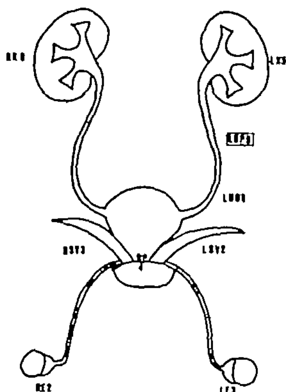


Fig 2 Results of inoculation into left intact ureter of 10 animals (group I) Figures indicate number of animals with histological tuberculous changes.

the upper part of the left ureter (LUP) In the group of animals with ligated ureter mucosal ulceration was apparent, the lumen of the ureter contained cell debris, granulocytes and tubercle bacilli and in the lamina propria there was granulocyte invasion and also epithelioid cell granulomas. In some of the animals of both groups, the tunica muscularis was intact, in others, there was epithelioid cell granulomas with central necrosis and tubercle bacilli in the muscle layer and even in the periureteral tissue. In the group of animals with non ligated ureter the three animals with uretero-hydronephrosis exhibited mucosal ulceration, while the mucosa was intact in the others. However in the ureteral wall and the peri ureteral tissue, epithelioid cell granuloma with areas of necrosis containing tubercle bacilli was often found.

In the region of the uretero-pelvic junction, the inflammatory changes were often localised to the peri-ureteral tissue alone.

In one animal (10) from group I changes were observed in the lumen of the distal region of the ureter namely mucosal ulceration together with granulocytes and tubercle bacilli.

On the other hand, in sections from both the proximal and distal parts of the ureter of animals in both groups, and corresponding to the macroscopically observed streaks on the ureteral wall, epithelioid cell granulomas were observed, usually extending around blood vessels in and around the lymphatics in the periureteral connective tissue (Figs. 4 5)

In the group of animals with ligated ureter, the left kidney was, in general, somewhat enlarged and of a soft consistence. No macroscopic changes were observed on the kidney surface. Histological examination revealed cell detritus, inflammatory cells and tubercle bacilli in the pelvis. The epithelium was part-

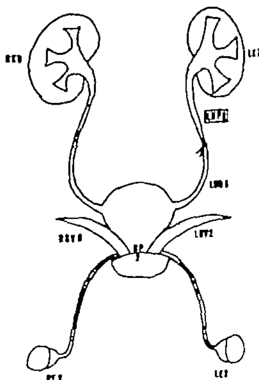


Fig 3 Results of inoculation into left ureter of 8 animals after ligation of ureter (group II) Figures indicate number of animals with histological tuberculous changes.



Fig 4 Section of distal part of ureter and peri-ureteral tissue 28 days after intra-ureteral injection of tubercle bacilli in an animal with ligated ureter (group II) Epithelioid cell granulomas and lymphocytes in and around lymphatics in peri-ureteral connective tissue. Htx-eosin × 40.

ly ulcerated, and epithelioid cell granulomas with small necrotic areas and tubercle bacilli were observed in the lamina propria. The collecting tubules were dilated, the epithelium was flattened and inflammatory cells with tubercle bacilli were sometimes found in the lumen (Fig 6) In only two animals from group II (12 and 15) epithelioid cell granulomas were found interstitially right up to the cortex and, in one animal from the same group (15) tubercles were found in the capsula fibrosa.

In the 3 animals with uretero-hydronephrosis (7 9 and 10) and also in two others (2 and 8) all belonging to the group of animals with intact ureter granulomas were found in the lamina propria of the pelvis up in the calyces. Tubercles were observed in the capsula fibrosa in animal 8. The left kidney was three times larger than normal in animal 10 being nodulous and completely infiltrated by tuberculous granulomas with necrotic areas and tubercle bacilli.

In sections from the right kidney inflammatory changes or tubercle bacilli were not discernible in any of the animals from the two groups.

The bladder exhibited no macroscopic changes in any of the animals, except animal 7 from group I in which the bladder was shrivelled and of firm consistence. Upon microscopical examination epithelioid cell granulomas with tubercle bacilli chiefly in the tunica adventitia, were found in animals from both groups.

In the group of animals with intact ureter mucosal alterations with ulcerations were observed in the bladder of two of the animals (animals 7 and 10) Granulomas with tubercle bacilli were also found in the bladder wall in animal 7 but not in animal 10

It applies to these two animals (7 and 10) and to a further three animals (1 2 and 8) that urine taken at autopsy from the bladder

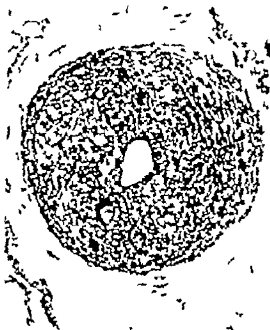


Fig 5 Enlargement of one of the epithelioid cell granulomas in fig. 4 with some typical Langhans' giant cells. Htx-eosin × 130

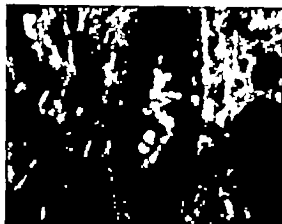


Fig 6 Macrophages containing tubercle bacilli in a collecting tubulus in an animal with ligated ureter (group II) Auramine-rhodamine fluorescent stain × 400

and cultured on Löwenstein-Jensen's medium contained tubercle bacilli they belonged all in the group of animals with intact ureter Specimens from a further two animals (14 and 18) among those with ligated ureter also gave a positive culture on Löwenstein-Jensen's medium.

Interstitial tuberculous prostatitis was never found isolated. The base of the bladder in such affected animals was invaded and gran



Fig 7 Section of prostate (P) urinary bladder (Bl) and ductus referens (DD) 28 days after intraureteral injection of tubercle bacilli into an animal with intact ureter (group I) Tuberculous granulation tissue interstitially partly surrounding secretory ducts in prostate. Htx-eosin × 40.



Fig 8 Section of prostate 28 days after intraureteral injection of tubercle bacilli into an animal with intact ureter (group I) Epithelioid cell granuloma adjacent to some small interstitial blood vessels. Htx-eosin × 100.

ulomatous inflammation spread out around the sperm ducts (Figs. 7 and 8)

Macroscopically there were no observable changes in the seminal vesicles. Histological examination revealed, however tuberculous infection in either one or both seminal vesicles in four animals among those with intact ureter (animals 1 2 4 and 5) as well as in two of those in the group of animals with ligated ureter (animals 11 and 12) The mucosa was intact in all animals. Epithelioid cell granulomas with tubercle bacilli were found in the tunica muscularis and adventitia.

There was spread to both epididymes in one animal in both groups (animals 1 and 13) while spread only to the left epididymis was observed in 2 animals (7 and 8) among those with intact ureter and in one animal (11) among those with ureteral ligature. A spread only to the right epididymis was found in one animal in either group (animals 2 and 16) The tuberculous changes were interstitial in the cauda epididymis or in the tunica albuginea at the boundary between the testis and the epididymis. In the corre-

sponding sperm duct no tuberculous changes or tubercle bacilli were found in the lumen, but in the periductal tissue they were often seen in and around the lymph spaces.

By gross inspection, lymphonodi iliaci para-aortales and/or renales were often found to be enlarged and microscopical examination revealed epithelioid cell granulomas in all animals of both groups. The granulomas were in part spread out, confluent, with necrotic areas and rich in tubercle bacilli.

The spleen was usually slightly enlarged and tubercle bacilli and epithelioid cell granulomas were demonstrated in 12 out of 18 animals. Cultures on blood taken at autopsy were negative in all animals.

DISCUSSION

Since genital tuberculosis is usually secondary to tuberculosis in the urinary tract, it seemed worthwhile to construct an experimental animal model to trace the paths of infection from the upper to the lower urinary tract and the male genital organs. It is, however hazardous to draw a parallel to the infectious process operative in man, as a relatively large bacterial dose applied to an non-immunized animal was used in the present study as well as in other animal experimental studies (*Zador et al* 1967 *Duchek* & *Winblad* 1973)

It is generally considered that tuberculosis in man spreads from the haematogenously infected kidney via the urinary (canalicular) routes with infected urine to the bladder and genitalia. In experiments upon rabbits with ligated ureter *Hansen* (1902) failed to find evidence for a spread of infection from the kidney to the lower urinary tract and genitalia, and concluded that the infection spread only via the urinary (canalicular) routes. Still, clinical experience (*Klosterhalfen* 1971) as well as earlier animal experimental observations (*Wildbolz* 1909 *Baurreisen* 1911) indicate that the peri-ureteral lymphatics *can* play an important role in the spread of infection.

Attempts to induce progressive kidney tuberculosis through direct injection into the kidney of guinea pigs are unsuccessful due to the kidneys resistance to tubercle bacilli (*Rich* 1944 *Birkhäuser* 1950) The kidney pelvis in guinea pigs is a difficult site for direct injection. It is, on the other hand easy to obtain access to the kidney pelvis if the inoculum is deposited via the ureter as in the present study

In the present investigation, the injected bacterial dose was shown to be sufficient to induce tuberculosis at the injection site in all animals, with one exception. Ureteral ligature was sufficient to prevent canalicular spread. In this group no changes were found in the mucosa in the lower part of the ureter Canalicular spread occurred in the group of animals with intact ureter but in this group as well as in the group of animals with ligated ureter spread occurred principally via the lymphatics in the peri-ureteral tissue. A probable explanation of this is, in contradistinction to Hansen s findings that spreading to the genital organs occurred in spite of ureteral ligature and injection of tubercle bacilli above the ligatures that the peri-ureteral tissue with its vessels was dissected free prior to ligaturing in the present study

A considerable uretero-hydronephrosis in the animals with ligated ureter was expected. Such a hydronephrotic kidney suffers extensive damage even in the absence of concomitant infection. A reduced kidney function and resistance brought about in this manner can explain why tuberculous changes occurred also in the kidney parenchyma in some of the animals with uretero-hydronephrosis. Similar arguments are valid in the case of the non-ligated animals with total or partial ureteral occlusion. Kidney tuberculosis involving one side *only* negative blood culture and lack of signs of miliary development speak against a haematogenous spread. Tuberculous granulomatous in the peripelvic lymphatics and interstitially in the kidney together with granulomas in the capsule are indications of a combined lymphatic and direct invasive spread of infection. This is supported by the finding that the streak formed spread in the tissue could be followed to the para-aortic

and renal lymph nodes. Lymphatic routes of spread to the kidney pelvis were indicated by *Walker* (1913) In one out of five guinea-pigs inoculated with tubercle bacilli in the urethra he found such bacilli in the lymphatics surrounding the upper portion of the ureter

In both groups in the present investigation, a spread to the urinary bladder occurred. Mucosal ulceration was found only in two animals, the ureter being intact in both. Urine culture was positive in these animals and a canalicular urinary spread appears likely In the other animals, the tuberculous changes were found mainly in the adventitia and tunica muscularis. In all probability the route of infection was via the distal ureteral lymphatics.

When the tuberculous infection has reached the bladder and the pelvic genitalia, spread to the scrotal genitalia can occur Spread to the epididymis occurred through the tunica adventitia of the sperm duct, which agrees with our earlier investigations (*Duchek* & *Winblad* 1973)

The conclusions to be drawn from these experiments on guinea pigs are that spreading of tuberculous infection from the kidney pelvis and proximal ureter occurs in either case whether ureters are intact or ligated. The spread of infection does not only occur canalicularly but also lymphatically When the tuberculous infection has reached the distal portion of the ureter or bladder there is rtl spread to the genitalia.

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RENAL TUMOURS IN CHILDREN

A Histological Evaluation

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Mäkinen, J. & Rapola, J. Renal tumours in children. A histological evaluation. Acta path. microbiol. scand. Sect. A 83 237–44 1975.

A ten-year series of renal tumours in children up to 14 years of age was collected from the files of the Finnish Cancer Register. After histological evaluation of the material, 74 cases were accepted as primary renal tumours. Fifty-eight of these were typical Wilms' tumours, three rhabdomyosarcomas, six foetal hamartomas and seven unclassified malignant tumours. Wilms' tumours were classified into three types on the basis of the histological differentiation. The total five year survival in the Wilms group was 50 per cent with no difference between patients over and under the age of one year. Of the subtypes of Wilms' tumours the sarcomatous type showed worse prognosis than average: only three out of 16 patients were alive after a five-year follow-up time. None of the patients with foetal hamartoma died of tumour although two fatalities, connected with the surgery, were recorded. The value of histological classification of Wilms' tumours and the importance of the recognition of the foetal hamartoma is emphasized.

Key words: Renal tumours; children; histology.

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Wilms tumour is one of the most common, solid and malignant tumours in children, exceeded in frequency only by neuroblastoma (*Klapproth* 1959, *Perez et al.* 1973, *Hesser & Cr ccy* 1932).

The prognosis of patients with Wilms' tumour varies in different reports, but it seems to be improving considerably in recent years, possibly due to the more accurate diagnostics and aggressive treatment.

The extent of the tumour's growth and the therapeutic procedures apparently influence the prognosis of the patients, and certain other biological factors also seem to be of prognostic significance. Some authors have indicated that the prognosis of the patient was better if the tumour was diagnosed and treated under the age of one year (*Favara et al.* 1968, *Wasman & Cooper* 1970). Some authors have recently paid attention to a congenital kidney tumour which differs from the classical nephroblastoma both clinically and morphologically. This tumour has been referred to as foetal hamartoma, congenital mesoblastic nephroma, fibroma of the kidney, metanephric hamartoma, and leiomyoma (*Bolande et al.* 1967, *Liban & Kozenitzky* 1970, *Richmond & Dougall* 1970, *Wasman & Cooper* 1970, *Wigger* 1969). *Bolande et al.* as well as other authors, have emphasized the benign nature of this tumour and suggest that it be distinguished from the classical malignant Wilms' tumour and that it be



Fig 1 Type I Wilms tumour showing a mixed pattern of well-differentiated tubular and mesenchymal components (H & E, × 400)

treated by simple nephrectomy alone (*Bolande et al* 1967, *Beckwith* 1970, *Kay et al.* 1966, *Wasiman & Cooper* 1970)

Apart from the foetal hamartoma, the prognostic significance of the histological structure of Wilms tumour is a controversial issue. *Hardwick & Stowens* in 1961 claimed there was a better prognosis in cases of highly differentiated nephronic structures than in cases of undifferentiated and sarcomatous histology. Similar results were obtained by *Knutrud* in the same year. Other investigations have not confirmed this postulation (*Marsden & Steward* 1969) but recently *Jereb & Sandstedt* (1973) reported significant correlation between the histology and prognosis of nephroblastoma patients.

We have analysed a ten-year retrospective period based on the files of the Finnish Cancer Registry. The material was studied histologically. An attempt was made to evaluate the prognostic value of different histological

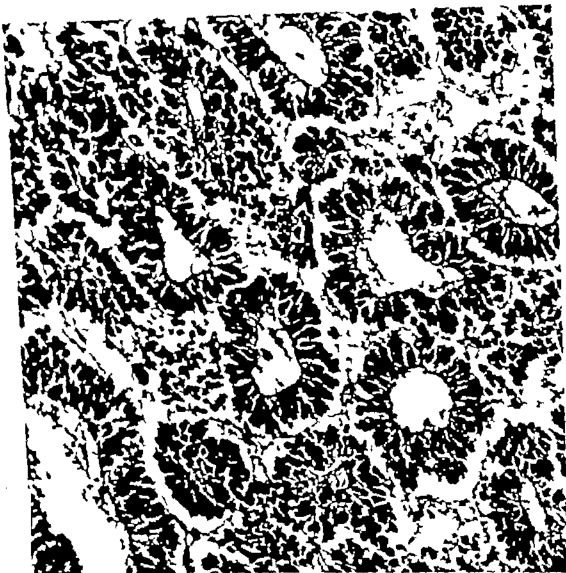


Fig 2 Type II Wilms tumour demonstrating poorly differentiated tubular structures (H & E, × 550)

tumour types with special emphasis on the foetal hamartoma. Correlation between the age of the tumour patients and survival was also sought.

MATERIAL AND METHODS

The material of 107 cases was collected from the reports on renal tumours in children up to 14 years age filed in the Finnish Cancer Registry between January 1 1958, to December 31 1967 In the same interval there were altogether 102,318 cancer cases registered in Finland, among them 1 454 cases (7 per cent) in children under 15 years. Thus the renal tumours represent about 13.6 per cent of all tumours occurring in children in the time interval mentioned.

The clinical data were obtained from records from the various hospitals at which the children were treated. In 39 cases only biopsy specimens were available In 26 cases both biopsy and autopsy specimens were examined. Histological slides and paraffin blocks (when needed) were obtained from the respective pathology laboratories.

The slides, stained with haematoxylin and eosin and/or the van Gieson method, were reviewed independently by both authors. Wilms' tumours

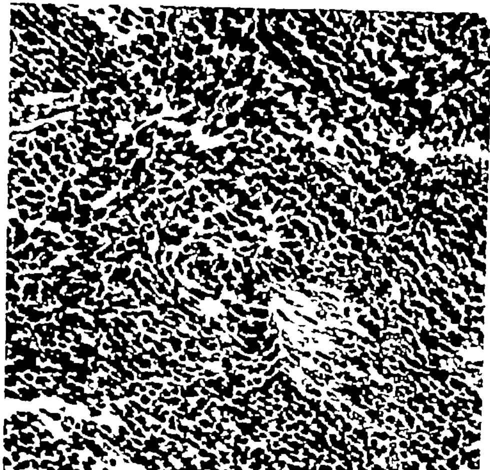


Fig 3 Type III Wilms tumour showing highly cellular spindle-celled sarcomatous neoplasm with polymorphism and mitotic figures (H & E, × 450)

(nephroblastomas) were considered to be cases showing typical epithelial and/or mesenchymal components, resembling metanephric blastema. Foetal hamartoma was a clearly separable group, showing an admixture of well-differentiated smooth muscle and fibrocytic fibres with numerous capillaries as described, among others, by *Bolande et al.* (1967) It appeared that this material consisted of other tumours as well and these were classified according to the conventional histological criteria.

Wilms' tumours were further classified into three types on the basis of the histological differentiation. Type I consisted of tumours showing a mixed pattern of tubular and mesenchymal components, all fairly well differentiated (Fig 1) Glomerulus-lik structures were often present. Type II showed poorly differentiated tubular structures with loss of cellular polarity and nuclear polymorphy. Mesenchymal components were very scanty in these tumours (Fig. 2) Tumours of type III were highly cellular usually spindle-celled sarcomatous neoplasms with only an occasional presence of tubular or other epithelial components. Cellular polymorphism and mitotic figures were frequent in this type of tumour (Fig 3) Three cases of typical embryonal type rhabdomyosarcomas were treated as a separate group of malignant tumours, although they might be considered as derivates of nephroblastomas.

Our initial purpose was to classify the tumours into five types according to the classification of *Hardwick & Stowens* (1961) but the variations between several slides of the same tumour made this impossible. The present classification is very close to the classification presented by *Jereb & Sandstedt* (1973) Twenty-two cases were not included in the final analysis because of insufficient clinical and/or morphological data. Thus the final series comprised 85 patients with histologically proven renal tumours occurring in children younger than 15

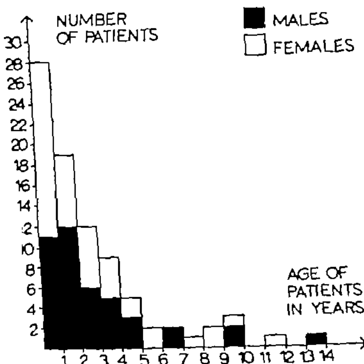


Fig 4 Distribution of the patients by age and sex.

RESULTS

Age and sex distribution There were altogether 40 males and 45 females in this material and 28 children were younger than one year (32.9 per cent) Fifty-nine children were under the age of three (79.4 per cent) The distribution of the patients in the different age groups is shown in Fig 4

Histological classification The majority of the cases (58) were typical Wilms' tumours as defined above (Table 1) There were as many as 11 neuroblastomas, probably originating from the adrenal gland of the same side and macroscopically giving an appearance of renal tumours. In fact it was possible to distinguish in some slides that the neuroblastoma ensheathed intact kidney Six cases represented typical foetal hamartoma. In seven cases the histological appearance differed so much from the usual nephroblastoma and other tumours described above that these were not classified at all. Some of them were probably lymphomas and some are epithelial tumours of uncertain cellular origin.

TABLE 1 *Distribution of the Tumours in the Different Histological Groups*

Type of tumour	Number of cases
Wilms tumour	58
Hamartoma	6
Rhabdomyosarcoma	3
Neuroblastoma	11
Unclassified mal. tu.	7
All cases	85

The majority of the Wilms' tumours belonged to type I, whereas the other two types were about equally frequent. The distribution of the cases of Wilms tumours in the different histological groups is shown in Table 2

Survival In Wilms tumours the total survival was 50 per cent. There was no difference if the patients under one year of age were separated into a group of their own seven out of 14 patients died and seven survived. The survival in different histological groups is shown in Table 3

TABLE 2 *Distribution of the Cases of Wilms Tumour in the Different Histological Subgroups*

Type of tumour	Number of cases
Type I	31
Type II	11
Type III	16
All Wilms tumours	58

TABLE 3 *Survival of Patients in the Different Histological Groups*

Type of tumour	Number of cases	Survived for 5 yrs
Wilms' tumour		
Type I	31	18
Type II	11	8
Type III	16	3
Hamartoma	6	4
Rhabdomyosarcoma	3	–
Neuroblastoma	11	2
Unclassified mal. tu.	7	1
All cases	85	36

Among six patients with foetal hamartoma none died of tumour. In this group two patients died: the one was a newborn boy who died right after surgery due to cardiac arrest and the other patient died due to septic infection during the postoperative period.

In the subtypes of Wilms tumour the mesenchymal, sarcomatous type showed a clearly worse prognosis than average. If we added the three cases of rhabdomyosarcoma to this group—all of whom died—the sarcoma represented the most grim prognostic group. The prognosis was equally gloomy in the other malignant non-Wilms tumours in the present material.

DISCUSSION

Large tumours confined to the kidney in children are easily interpreted as Wilms' tumours or nephroblastomas unless an accurate histological study is made (*Black & Ragsdale* 1968). Because the histological pattern varies greatly from relatively well-differentiated embryonic kidney-like tissue to anaplastic tumour even a histological assessment may be difficult. Thus we were surprised to find so many neuroblastomas in the present material. Large neuroblastomas of the adrenal gland may however grow so that it is impossible to distinguish them macroscopically from the Wilms tumour.

Histological assessment of primary renal tumours is also essential. The present study confirms other reports (*Bolande et al.* 1967, *Burkholder et al.* 1970, *Kay et al.* 1966, *Wigger* 1969) according to which the foetal hamartoma is a distinctive and probably a benign condition (Fig. 5). To our knowledge only one case is on record in which the tumour recurred after initial surgery (*Fu & Kay* 1973).

The recognition of foetal hamartoma gains more importance from the fact that it is often found in newborns on whom treatment with the Actinomycin D currently used in the therapy of Wilms tumours may be fatal (*Pochedly et al.* 1971). The percentage of foetal hamartomas was 8 in the present material after subtracting the neuroblastomas. In previous reports its frequency has been reported to range from 0–5 per cent (*Bolande et al.* 1967, *Wigger* 1969).

The present material did not reveal any prognostic advantage of the young age of the patients with typical Wilms tumour as has been claimed by some authors (*Lattimer et al.* 1959, *Margolis et al.* 1973, *Perez et al.* 1973).

The advantage of young age has not appeared in other materials (*Platt & Linden* 1964) and recent, carefully analysed series comprising 112 nephroblastoma cases gave the same conclusion (*Jereb & Sandstedt* 1973). The same investigators found (*Jereb* 1973) however that young age had a favourable effect on survival in the small percentage of patients initially treated by irradiation whose tumours were locally limited. It is possible that the reports of better prognosis in very young age groups are due to the over representation of foetal hamartomas in

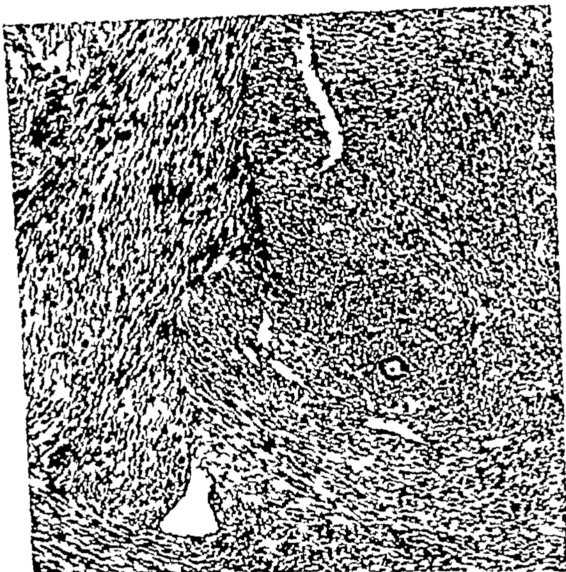


Fig. 5. Typical histological pattern of *foetal* hamartoma of the kidney with occasional included tubular structure (H & E, × 160)

these groups of patients (*Bolande et al.* 1967)

We are well aware that the prognostic significance of a histological classification which does *not* take into account other factors such as the extent of the initial tumour growth at the time of surgery and the mode of therapy has serious limitations. The difference in prognosis between well-differentiated Wilms tumours (type I and II survival 62 per cent) and sarcomatous types (type III and rhabdomyosarcomas, survival 15 per cent) suggest, however that the histological classification of Wilms' tumours is of some clinical value.

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THE BASEMENT MEMBRANE AND LOBULAR CARCINOMA IN SITU OF THE BREAST

A Light Microscopical Study

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Andersen, J A. The basement membrane and lobular carcinoma in situ of the breast. A light microscopical study Acta path. microbiol. scand. Sect. A, 83 245–250, 1975

The basement membrane (BM) was examined by light microscopy in cases of lobular carcinoma in situ of the breast (LCIS) employing the following stains haematoxylin-eosin, van Gieson-Hansen, PAS, colloidal-iron-PAS, and reticulin. The BM was non-intact in 12 out of 26 investigated cases of LCIS when reticulin staining was applied, but appeared non-intact in about 70 per cents with the other staining methods. On the other side control areas of the BM were intact in 25 out of 26 cases with reticulin stain, but in only about half of the cases with other stains. It is concluded that reticulin staining is the most suitable in evaluating the BM in LCIS and that more or less pronounced defects of the BM in LCIS cannot be considered evidence of imminent invasion. Although the overall occurrence of invasive breast carcinoma was significantly increased in 9 cases with epithelial cell protrusion through the BM this feature should be investigated further before it is allowed to affect therapeutic decisions.

Key words Breast lobular carcinoma in situ basement membrane.

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When studied by light microscopy the basement membrane (BM) in breast tissue is seen to be about 1 micron in thickness. It consists mostly of an amorphous protein polysaccharide ground substance and fibrils as well as, just around the glandular structures, of a filamentous textured so-called basal lamina (BL) which is 500–800 Å in thickness and is visible only under the electron microscope (*Bloom & Fawcett* 1968)

When *Broders* (1932) introduced the term carcinoma in situ, the BM was mentioned as being the border through which malignant epithelial cells had to penetrate in order to form a carcinoma possessing the ability to metastasize.

Whereas ultrastructural studies of the BL and the BM in breasts with LCIS are included in several recent investigations (*Carter et al.* 1969 *Schäfer & Bässler* 1969 *Ozzello* 1971 *Ozzello* 1971 *Tobon & Price* 1972) only a few light microscopical studies are available concerning the BM in carcinoma in situ in general (*Ozzello & Speer* 1958 *Ozzello* 1959 *Grossman & Rigaud* 1972) It

has not been possible in the literature to find any special reports concerning the BM in LCIS.

MATERIAL AND METHODS

The material consisted of 52 cases of LCIS without simultaneous invasive carcinoma as described earlier (*Andersen* 1974)

Microscopical slides from the tissue blocks available were stained in the following ways: haematoxylin-eosin, van Gieson-Hansen, PAS, colloidal-iron-PAS (aqueous solution of periodic acid) and reticulin (Foot furthermore Wilder in 14 cases)

Under the light microscope the BM is quite well defined, but the borders can be blurred, varying from one staining method to the other. Therefore, the thickness of the BM was not measured quantitatively but estimated as follows only a BM which was clearly either thinner or thicker than the normal BM was classified as such, and complete absence of BM in smaller or larger areas indicated that its presence was not even suspected.

Protrusion of cells means that one, and possibly several epithelial cells are situated outside the normal outline of the ductules, although still in contact with the remaining epithelial cells, and that the BM presents defects corresponding to the protruded cells.

In each case and separately for each method of staining the BM was checked of at least 10 adjacent lobules, in which development of LCIS was not suspected. Only slides were evaluated in which the individual staining method revealed areas with LCIS.

RESULTS

The BM appeared as a dense, almost amorphous layer of acellular material around the lobular ductules. The BM and the epithelium were subdivided by a discontinuous layer of flattened myoepithelial cells. In the slides stained with haematoxylin-eosin the BM was eosinophilic and, because of the dense structure, almost hyaline. In most cases, therefore, it was easy to distinguish the BM from the loose intralobular stroma, but if the stroma was firmly fibrous, it could be difficult to discern the BM. In the slides stained with van Gieson-Hansen stain, the BM was red, almost the same colour as the surrounding connective tissue, but there was a distinct contrast between the BM and the epithelium. In the PAS and colloidal-iron-PAS stained slides, the BM was bright purple-red. The contrast between the BM and the connective tissue was in many cases vaque with ordinary PAS staining but most often the contrast to the blue or bluish-red stroma in colloidal-iron-PAS stained slides was good. Because of the distinctly PAS-positive granules and the concomitant accentuation of the cellular border in LCIS (*Andersen* 1974) the contrast between the BM and the epithelium in LCIS was, however not always very clear. In some cases the stainability of the BM varied, being blue with colloidal-iron-PAS staining and with ordinary PAS-staining having exactly the same faint reddish hue as the stroma.

The best presentation of the BM, and that showing the clearest contrast, was obtained by reticulin staining (Fig 1) Thus it was possible to observe distinctly tangential sectioning of the BM a faint black diffuse structure was visible, with heavily black stained fibrils running in all directions.

Generally the thickness of the BM in LCIS was normal in 6 cases, thicker in 4 cases, and thinner than normal in 39 cases. In 3 cases the thickness varied greatly

In many cases the BM was not well defined. It could be completely absent in smaller or greater area, or it could be undiscernible with a particular staining method (Fig 2) The results of this part of the study appear in Table 1 Apart from the reticulin staining the other stains gave fairly uniform results. Hence the BM did not seem to be intact in about 70 per cents of the LCIS cases and the same applied to a little less than 50 per cents of the control areas. However there was no doubt that the BM in general was more poorly defined in the LCIS areas than in the controls, where it was mostly and only very limited areas that the BM was apparently not intact.

Fig 1 LCIS with a diffusely thin, but well defined and in all areas intact BM. Reticulin 456 ×

Fig 2 LCIS without BM in larger areas. Reticulin 456 ×

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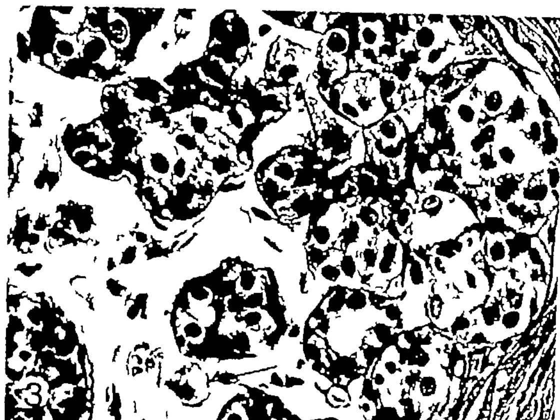


Fig 3 LCIS with protrusion of individual cells (arrows) Haematoxylin-eosin 456 ×

TABLE 1 *Evaluation of the BM Using Various Staining Methods in Cases of LCIS*

Staining method	LCIS		Control areas	
	BM intact	BM non-intact	BM intact	BM non-intact
Haematoxylin-eosin n* = 51	15	36	27	24
Van Gieson-Hansen n = 44	10	34	23	21
PAS n = 31	9	22	18	13
Colloidal-iron-PAS n = 20	6	14	11	9
Reticulin n = 26	14	12	23	3

*n = cases investigated in the individual staining method.

The reticulin stain revealed practically an equal number of cases of LCIS with intact BM (14 cases) as with non intact BM (12 cases) The overall occurrence of invasive breast carcinoma (previous and subsequent) in these two groups was 5 and 6, respectively On the contrary the BM in the control areas was in the majority of cases intact.

Protrusion of cell(s) was suspected in 14 cases, but in only 9 could this suspicion be maintained with any certainty (Fig 3) In the remaining cases tangential sectioning, in particular of longitudinally cut ends of ductules, was most likely Seven of the 14 cases with primarily suspected protrusion of cell(s) were cases with previous or subsequent invasive breast carcinoma. In the 9 cases with maintained suspicion of protrusion of cells, the overall occurrence of invasive carcinoma was 6 (2 previous and 4 subsequent, and of

these 4 3 ipsilateral and 1 contralateral) This overall occurrence of invasive breast carcinoma in cases with cell protrusion is significant ($P<0.05$)

DISCUSSION

Ozzello & Speer (1958) who employed the colloidal-iron-PAS method, found the BM evident in both duct cell noninvasive carcinoma and LCIS and present in normal glands as well as in cases of mammary dysplasia (non-neoplastic lesions) In the last mentioned cases, changes such as thinning, swelling, and poor definition of the BM were seen.

Ozzello (1959) who also used the colloidal iron-PAS method, demonstrated disruption or complete absence or colour changes of the BM in 17 out of 18 cases of intraductal carcinoma but did not find these observations in benign lesions. He presumed that a poorly defined or disrupted BM was the first detectable evidence of invasion.

Grossman & Rigaud (1972) used the haematoxylin-eosin stain and gained nothing by using PAS, reticulin or Mason trichrome staining in the assessment of the BM. They found an intact BM (their "eosinophilic membrane") in intraductal carcinoma and LCIS and they found the BM to be present in all benign areas of the breast tissue controls. On the other hand, they found total absence of BM in the majority of invasive carcinomas, but in a very few cases partial absence only

That the results of the present study are in disagreement with in particular the two last studies, are evident. As regards *Grossman & Rigaud* (1972) the disagreement may be caused mainly by the fact that these authors studied the BM in invasive carcinoma and used the adjacent breast tissue as control. In these border areas there will often be pronounced inflammatory reactions, which will generally produce pronounced thickening of the BM At the same time there will be remnants of ductules, which will also frequently have a very thick and distinct BM Although to a lesser degree, the same applies to the disagreement with the results obtained by *Ozzello* (1959) It must be borne in mind, however that Ozzello's study comprised cases with intraductal carcinoma.

From the majority of the ultrastructural studies concerning LCIS it appears that the BL were intact (*Carter et al.* 1969 *Schäffer & Bässler* 1969 *Tobon & Price* 1972) However *Ozzello* (1971) emphasized that protrusion of cells was rather frequent, and he considered this feature as evidence of early invasion. He found this frequently in intraductal carcinoma, but less frequently in LCIS. Nevertheless, he stated that protrusion through the BL could also be seen in non-neoplastic conditions, e.g in vascular endothelium.

Light microscopical studies will rarely show protrusion of cells, and the demonstration is difficult and rather inaccurate. Although the overall occurrence of invasive carcinoma seems to be rather high in the present study I am of the opinion that the results should be submitted to further trials before therapeutic decisions are affected.

CONCLUSIONS

1 Reticulin staining is the conventional histological staining method which is best suited for the evaluation of the BM by light microscopy in breast tissue.

2. Larger or smaller defects of the basement membrane in LCIS of the breast cannot be considered as evidence of an imminent invasion.

3 The observation of protrusion of cells by light microscopy is a rather rare phenomenon in lobular carcinoma in situ. The appearance seems to show a significant increased overall occurrence of invasive breast carcinoma. However this feature should be investigated further before it is allowed to affect therapeutic decisions.

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QUANTITATIVE STUDIES OF THE RENAL CORPUSCLES III

The Influence of Post Mortem Delay before Taking Renal Tissue Samples and of the Duration of Tissue Fixation

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sen, Hanberg F Quantitativ studies of the renal corpuscles III The influence of post mor delay before taking renal tissue samples, and of the duration of tissue fixation. Acta path. obiol scand. Sect. A 83 251–258, 1975

parative quantitative studies of glomeruli in biopsies of the guenon monkey kidney were e to giv a numerical measure of the effect of varying periods of fixation of the biopsy e in Carnoy's fluid (1–2–24 hrs.) and of the effect of progressive post mortem delay re renal tissue samples were taken (2–4–6–12 hrs.) Additional aims were to determine ible quantitativ differences between subcapsular intermediate and juxtamedullary nerul, and to determine whether the glomerular area should be determined with reference he inner aspect of Bowman capsule (total area) or to tangents laid at the top of the llary loops (corrected total area) The quantitativ methods used were total and dif ntial counts of glomerular nuclei by light microscopy and point counting for determination glomerular and mesangial areas. Statistical evaluation was made by a two-way analysis ariance. The differential counts of nuclei were not affected by duration of fixation, nor post mortem delay up to 12 hrs. before tissue samples were taken. The mesangial area ressed as a percentage of total area was significantly lower two hours post mortem, while al nuclear concentration was significantly lower t time 0 and two hours post mortem n at four six and 12 hours post mortem. Total nuclear concentration increased as dura n of fixation was increased, probably a reflection of tissue shrinking. Differences in nuclear centration were also noted at different levels of the renal cortex. These differences were ly pparent when the glomerular area was determined with reference to the inner aspect of wman capsule and disappeared when the corrected total area was used. It is concluded at duration of tissue fixation and post mortem delay before tissue samples were taken both fect method sensitivity as reflected by some, not all, of the parameters measured. The merical differences in the parameters were not large, however and it will be the specific dy which will determine whether variable fixation times and post mortem delay in ob ning tissue samples is acceptable. As most renal biopsies contain both subcapsular and ctamedullary glomeruli there is probably no advantage in using the corrected glomerular area stead of the total glomerular area limited by Bowman capsule.

ey words Renal corpuscles post mortem delay fixation duration.

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Several authors have reported the practic ability of a quantitative study of glomeruli by light microscopy of silver or periodic-acid Schiff haematoxylin stained kidney preparations. These studies comprise total and differential counts of glomerular nuclei including determination of both mesangial and total glomerular areas (*Iidaka et al.* 1968, *Wehner* 1968a, *Wehner* 1968b, *Wehner & Anders* 1970 *Wehner et al.* 1970 *Kawano et al.* 1971a, *Hanberg Sorensen* 1972) Two methods have been used either camera lucida drawings using planimetric determination of the areas, or direct microscopy for total and differential nuclear counts, using point counting for determination of the mesangial areas. Each method has its adherents, and despite comparative studies, there is no agreement whether one or the other method should be preferred (*Kawano et al.* 1971b *Hanberg Sorensen & Ledet* 1972)

Provided that socalled central sections of the glomeruli are examined, it appears from a previous study of normal renal tissue that about 10 glomeruli should be scrutinized (*Hanberg Sorensen & Ledet* 1972)

The ultimate aim of such studies is the development of a quantitative method for use in diagnostic renal pathology and to this end it is desirable that the importance of the duration of tissue fixation should be determined. Similarly the validity of post -tem changes after any delay should be

The main objects of this study were thus 1) to compare findings in preparations from normal tissues kept in the fixation medium (Carnoy's fluid) for different periods of time, and 2) to compare findings in normal tissues from which preparations were made at varying intervals after the death of the donor The study also gives 3) a supplement to an earlier comparative study of quantitative findings in subcapsular and juxtamedullary glomeruli (*Hanberg Sorensen* 1972) and considers 4) whether the glomerular area should be determined using the inner limit of Bowman's capsule as the boundary or whether by laying tangents, the area should be determined with reference to the top of the capillary loops.

MATERIAL AND METHODS

The chosen donor of renal tissue was the guenon, a monkey with glomeruli which under light microscopy study are seen to resemble closely those of man. Bilateral nephrectomy was performed on a healthy four years old guenon under Leopental anaesthesia. Tissue samples were taken immediately at random, for the fixation study and for the base study of the post mortem changes (time 0). Thereafter the kidneys were packed in cloths damped with physiological saline and kept for six hours at room temperature and for a further six hours at 6 C in an attempt to mimic the temperature changes which occur in the tissues after death in hospital. Random tissue samples were taken for the study of time post mortem dependent changes at two, four six and 12 hours after nephrectomy All samples were approximately 1 mm thick, wedges of renal tissue. The samples for the study of post mortem changes were fixed in Carnoy's fluid for one hour while the samples for the fixation study were kept in the same fluid for one two, 24 and 72 hours. Paraffin wax sections were cut on a microtome adjusted to 2 μm, and stained with periodic-acid-Schiff-haematoxylin

The quantitative methods are previously described in detail (*Hanberg Sorensen & Ledet* 1972) For this reason it should only be mentioned that the method used in this study is direct microscopy for total and differential counts of the glomerular nuclei, and point counting for the determination of mesangial area, total glomerular area, and the corrected total area The total glomerular area is taken to be the area within Bowman's capsule, while the "corrected total area is the area defined by tangents laid at the top of the capillary loops (*Østerby* 1973) The tangents were laid in such a way that the inner angles between tangents were <180 A Leitz Ortolux microscope was used with a mirror for projection The objective was a Leitz A 45/0,65 Differential counts were made at × 500 enlargement, and area determinations at × 1035 Point spacing in the area determinations was 17 μm

RESULTS

In renal tissue specimens taken at time 0 and fixed for varying periods of time (1 2 24 hours) as well as in specimens with a constant period of fixation and a variable post mortem delay before samples were taken (0,

TABLE 1 *Mean Values of Glomerular Parameters*

		Post mortem delay in hrs.					
		0	2	4	6	12	Mean
Mesangial area per cent of total area	S	16.06	13.34	15.81	15.55	16.33	15.42
	IM	13.98	13.93	14.45	15.22	15.77	14.67
	J	13.55	12.24	13.93	15.80	15.14	14.49
	Mean	14.47	13.17	15.41	15.52	15.75	14.86
Mesangial area per cent of corrected total area	S	20.69	17.42	19.27	20.68	21.94	20.00
	IM	19.48	17.86	18.95	19.84	20.07	19.24
	J	19.45	16.20	19.48	20.52	19.54	18.99
	Mean	19.87	17.16	19.23	20.28	20.32	19.41
Total nuclei pr 1000 µm³ of total area	S	10.87	10.08	13.12	11.81	12.03	11.58
	IM	10.3	11.03	12.92	12.02	12.61	11.78
	J	9.67	10.54	13.97	12.41	12.91	11.90
	Mean	10.29	10.55	13.34	12.08	12.52	11.75
Total nuclei pr 1000 µm³ of corrected total area	S	15.26	15.18	15.98	13.74	16.15	15.26
	IM	14.36	14.15	16.95	15.64	16.09	15.43
	J	14.02	13.92	17.11	18.01	16.64	15.54
	Mean	14.55	13.74	16.68	15.79	16.29	15.41
Mesangial nuclei pr. 1000 µm³ of mesangial area	S	19.34	20.32	20.01	20.24	20.11	20.00
	IM	19.80	19.78	21.89	20.08	20.67	20.45
	J	18.90	20.37	21.65	21.06	21.06	20.60
	Mean	19.35	20.16	21.18	20.46	20.61	20.35
Endothelial nuclei per cent of total nuclei	S	42.07	44.82	44.64	45.25	44.55	43.86
	IM	42.45	45.58	44.32	45.28	43.22	45.73
	J	43.79	47.15	46.53	48.56	45.24	46.25
	Mean	42.76	45.78	45.16	45.03	44.34	44.62
Epithelial nuclei per cent of total nuclei	S	31.75	28.30	31.26	30.39	28.15	29.97
	IM	30.71	29.69	31.15	31.15	30.95	30.73
	J	29.87	29.14	28.79	4 99	29.90	28.53
	Mean	30.77	29.04	30.40	28.84	29.67	29.75
Mesangial nuclei per cent of total nuclei	S	26.18	26.87	24.09	26.26	27.28	26.14
	IM	26.81	24.92	24.51	23.44	25.82	23.50
	J	26.33	23.70	24.67	26.44	24.84	25.19
	Mean	26.44	25.16	24.42	26.05	25.98	25.61

Varying combinations of post mortem delay before sampling of renal tissues/localization of glomeruli. S = subcapsular IM = intermediate, J = juxtamedullary

2 4 6 12 hours) the following parameters were examined mesangial area as a percentage of the total glomerular area, mesangial area as a percentage of the corrected total area, total number of nuclei per 1000 mμ of the total glomerular area, total number of nuclei per 1000 mμ of corrected total area, mesangial nuclei per 1000 mμ of mesangial area, mesangial nuclei as a percentage of total nuclei, endothelial nuclei as a percentage of total nuclei and epithelial nuclei as a percentage of total nuclei. Referring to each

TABLE 2. *Comparison of Mean Values of Glomerular Parameters at Different Sites (I-III-I), Related to the Time Post Mortem of Which the Tissue Samples were Taken (0 2 4 6 12 hr)*

	Interaction	F-ratios Site avge.	Post mortem avge
Mesangial area per cent of total area	1.73	3.10*	8.83†
Mesangial area per cent of corrected total area	0.70	2.24	8.96†
Total nuclei pr 1000 μm² of total area	0.86	0.4	16.44†
Total nuclei pr 1000 μm² of corrected total area	1.29	0.47	21.56†
Mesangial nuclei pr 1000 μm² of mesangial area	0.71	1.01	2.81
Endothelial nuclei per cent of total nuclei	1.64	4.40*	3.15*
Epithelial nuclei per cent of total nuclei	1.62	4.87*	1.3
Mesangial nuclei per cent of total nuclei	0.80	1.08	1.85

Statistical evaluation two-way analyses of variance for each parameter The calculated F-ratios are appended

* significant ($p<0.05$)
§ significant ($p<0.01$)
† significant ($p<0.001$)

of the above mentioned times for post mortem sampling and each of the fixation times, 3 tissue specimens were examined. In each of these 3 specimens, the quantitative examination was made in central sections of 10 glomeruli. In the first sample sections of the 10 glomeruli closets to the capsule (subcapsular) were studied in the second sample the examination was made in the 10 glomeruli placed most centrally between the capsule and medulla (intermediate) and in the third, the 10 glomeruli closest to the medulla (juxtamedullary) The number of 10 glomeruli was chosen because earlier studies (*Hanberg Sørensen & Ladet* 1972) of central and peripheral glomerular sections showed that the SEM of all parameters was ≦5 per cent of the mean when 10 glomeruli from central sections were studied. This proved to be true also in studies of the guenon kidney

The statistical evaluation of the average parameter values was conducted as a two-way analysis of variance for each individual parameter using duration of fixation or time post mortem as the one allotment criterion, and site of sampling (subcapsular intermediate, juxtamedullary) as the other The prerequisites of the analysis of variance that the variables were normally distributed were fulfilled for every parameter (*Hanberg Sørensen* 1972) The prerequisite of homogeneity of variance among the variables was studied by application of Bartlett's test, and the variances could not be regarded as heterogenous at a significance level of 1 per cent.

Post mortem changes. Table 1 gives the mean values of the quantitative glomerular parameters at the respective points in time after death, and at each of the three localizations of the glomeruli. Table 2 gives the results of the statistical analyses. It appears from the tables 1) that the mesangial area as a percentage of both total and corrected total areas was significantly smaller two hours

TABLE 5 *Mean Values of Glomerular Parameters*

		Duration of fixation in hrs.			
		1	2	24	Mean
Mesangial area per cent of total area	S	16.06	14.46	13.61	14.71
	IM	13.98	12.30	14.32	13.53
	J	13.35	12.06	15.48	13.63
	Mean	14.47	12.94	14.47	13.96
Mesangial area per cent of corrected total area	S	20.69	19.06	17.16	18.97
	IM	19.48	16.62	18.08	18.08
	J	19.45	16.58	20.51	18.84
	Mean	19.87	17.42	18.52	18.62
Total nuclei per 1000 μm^2 of total area	S	11.87	11.26	12.46	11.87
	IM	10.32	10.56	12.38	11.09
	J	9.67	9.70	11.88	10.42
	Mean	10.62	10.51	12.25	11.13
Total nuclei per 1000 μm^2 of corrected total area	S	15.26	14.84	15.76	15.29
	IM	14.36	14.23	15.63	14.74
	J	14.07	13.38	15.71	14.37
	Mean	14.55	14.15	15.70	14.80
Mesangial nuclei per 1000 μm^2 of mesangial area	S	19.54	20.26	21.00	20.20
	IM	18.80	21.06	21.14	20.67
	J	18.90	20.68	20.81	20.15
	Mean	19.35	20.67	20.98	20.33
Endothelial nuclei per cent of total nuclei	S	42.07	41.76	45.19	43.01
	IM	42.46	45.34	45.80	43.87
	J	43.79	42.66	44.91	43.78
	Mean	42.76	43.25	44.63	43.55
Epithelial nuclei per cent of total nuclei	S	31.75	32.20	31.85	31.93
	IM	30.71	30.23	31.81	30.92
	J	29.87	31.72	28.10	29.90
	Mean	30.77	31.39	30.58	30.92
Mesangial nuclei per cent of total nuclei	S	26.18	26.03	22.94	25.05
	IM	26.81	24.39	24.35	25.18
	J	26.35	25.61	26.98	26.31
	Mean	26.44	25.34	24.75	25.51

Varying combinations of duration of fixation/localization of glomeruli.
S = subcapsular IM = Intermediate. J = juxtamedullary

post mortem ($p<0.001$) and 2) that the total nuclear count per 1000 $m\mu^2$ total and corrected total areas was significantly lower at time 0, and at two hours post mortem ($p<0.001$) 3) There was weakly significant difference between the average differential counts for endothelial and epithelial nuclei at each localization ($p<0.05$) and a correspondingly lower endothelial nuclear percentage of the total nuclear count at time 0

Different durations of fixation. Table 5

TABLE 4 *Comparison of Average Values of Glomerular Parameters at Different Sites (I–III) Related to Varying Duration of Fixation (1–2–24 hrs.)*

	F-ratios		
	Interaction	Site argr.	Fixation argr.
Mesangial area per cent of total area	4.84‡	3.67*	6.72‡
Mesangial area per cent of corrected total area	4.09‡	1.26	7.74†
Total nuclei pr 1000 μm^2 of total area	1.20	9.53†	17.03†
Total nuclei pr 1000 μm^2 of corrected total area	0.77	2.84	8.62†
Mesangial nuclei pr 1000 μm^2 of mesangial area	0.76	2.88	25.40†
Endothelial nuclei per cent of total nuclei	1.62	0.53	2.19
Epithelial nuclei per cent of total nuclei	1.61	2.91	0.50
Mesangial nuclei per cent of total nuclei	1.81	1.45	2.22

Statistical evaluation: two-way analysis of variance for each parameter. The calculated F-ratios are appended.

* significant ($p<0.05$)
‡ significant ($p<0.01$)
† significant ($p<0.001$)

gives the mean values of the quantitative glomerular parameters after the respective durations of fixation, and at each of the three localizations of the glomeruli. Table 4 gives the results of the statistical analyses. It can be concluded: 1) that the average total nuclear counts per 1000 $m\mu$ total area at the ... localizations differ significantly from each other ($p<0.001$) but there were no significant differences when nuclear counts were expressed per 1000 $m\mu$ corrected total area. 2) Nuclear counts per 1000 $m\mu$ total area, and nuclear counts per 1000 $m\mu^2$ corrected area were significantly higher after 24 hours' fixation ($p<0.001$). 3) Mesangial nuclear counts per 1000 $m\mu$ mesangium were significantly lower after one hour's fixation ($p<0.001$). 4) Mesangial areas as a percentage of both total and corrected total areas evidenced a significant interaction between the two allotment criteria. This interaction is difficult to explain, and the significant difference in the mean values cannot therefore be meaningfully interpreted. 5) There were no significant differences in the differential counts.

Study after tissue fixation for 72 hours was also intended but at this time the glomerular contours were not clear and the individual structures were thus not amenable of quantitative study.

DISCUSSION

A requirement of precise fixation time for renal biopsies can give rise to practical difficulties in the laboratories. If a quantitative method therefore is to be used in evaluations of renal biopsies it is desirable to identify the importance of the duration of fixation on the quantitative parameters. It can then be determined whether the calculated variation in the quantitative parameters is acceptable or alternatively that fixation time must be strictly controlled within narrow limits.

Similarly as it in some cases might be ne-

cessary to study renal tissue taken post mortem, it is desirable to evaluate the effect of any delay with consequent progression of post mortem tissue changes, on the quantitative parameters.

The studies here reported suggest that the differential nuclear counts are unaffected by duration of fixation in Carnoy's fluid, and also unaffected by the time of biopsy taking. The single, weakly significant variation in the endothelial nuclei in the post mortem study can hardly be important. Mesangial area as a percentage of total area was significantly lower at two hours post mortem, and total nuclear concentration was significantly lower at time 0 and two hours post mortem. *Iidaka et al.* (1968) compared findings in autopsy tissue with those in renal biopsies taken from living subjects, and found significantly lower mesangial area percentages in the autopsy tissue samples, but no differences in nuclear density nor in differential counts of nuclei. It is not clear why this should be so, nor why there is a discrepancy between the findings in the present study and those obtained in *Iidaka et al.*'s study but it seems probable that different degrees of filling and collapse of capillaries give rise to variation in mesangial area percentages, and in total nuclear density.

The fixation study indicated that nuclear density increased as fixation time in Carnoy's fluid is prolonged. The increasing nuclear density may reasonably be taken to reflect a shrinking phenomenon. Possible changes in mesangial area percentage due to a prolonged fixation could not be demonstrated because of interaction between the two allotment criteria in the two-way analysis of variance.

The observed changes dependent on duration of fixation and post mortem delay before tissue sampling were not large although statistically significant. The ultimate standards established in the individual laboratories will depend partly on the facilities there and partly on the nature of the particular problem to be studied.

It is encouraging that the differential count of nuclei probably the best quantitative parameter is not affected by differences in duration of fixation of tissue, nor by post mortem delay in sampling of tissue. Of course, if variable fixation times are chosen these should apply to both normal and test material.

In a previous study of human tissue (*Hanberg Sorensen* 1972) the named parameters indicated no difference between subcapsular and juxtamedullary glomeruli, although the largest glomeruli were found juxtamedullarily as also noted by *Elias & Hennig* (1967). *Hanberg Sorensen*'s previous study concerned only two tissue samples, and for this reason the present study also serves as a supplement. Only one highly significant localization dependent difference between glomeruli was noted—the nuclear count per 1000 $m\mu^2$ total area in the fixation study. There was no such complementary findings in the post mortem study. This may suggest that in some locations there is a different degree of expansion of Bowman's capsule at subcapsular and juxtamedullar sites, because the difference is no longer apparent when the nuclear counts are alternatively expressed per 1000 $m\mu$ corrected total area.

Østerby (1973) determined the glomerular area with reference to the top of the capillary loops in her electron microscopy studies, in the same way as *Iidaka et al.* (1968) in light microscopy studies of a limited number of glomeruli. The latter authors found a constant difference between nuclear counts per unit glomerular area dependent on whether that area was determined with reference to the inner margin of Bowman's capsule, or to the top of the capillary loops.

The study here reported suggests that there can be a localization dependent difference between nuclear concentrations in subcapsular and juxtamedullary glomeruli when total area is calculated with reference to the inner margin of Bowman's capsule but not when the reference is the top of the capillary loops i.e. the corrected total area. This might suggest that the parameter most suitable for use in a quantitative study is the corrected total

area. As, however renal biopsies most often contain both subcapsular and juxtaglomerular glomeruli, the sensitivity of the method will probably not be essentially increased if the corrected area is used instead of the area limited by Bowman's capsule. In this connection it should also be considered that determination of the corrected total area is more time consuming

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HEALING OF A CRUSH INJURY IN RAT STRIATED MUSCLE

1 Description and Testing of a New Method of Inducing a Standard Injury to the Calf Muscles

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Järvinen, M. & Sorvari, T. Healing of a crush injury in rat striated muscle. 1 Description and testing of a new method of inducing a standard injury to the calf muscles. Acta path. microbiol. scand. Sect. A, 83 259-265 1975

In this report an apparatus is described which can quickly and reliably induce a standard crushing injury in a desired location of rat calf muscle without tearing the skin. To test the constancy of injury caused by the apparatus, both hind legs of rats were traumatized. Two days after traumatization, the rats were killed and the hind legs were inspected macroscopically and microscopically following prior formalin fixation. The breaking strength of the muscles was measured immediately after traumatization. In every case, the injury totally penetrated the medial head of the gastrocnemius muscle, but never damaged the whole jumping complex of muscles (gastrocnemius, plantaris and soleus). Both the location and the dimensions of the wound as well as the breaking strength of the injured muscles remained inside such narrow limits that the trauma can be regarded as constant.

Key words: Crush injury experimental striated muscle healing.

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The response of striated muscle tissue to crushing injury has been the subject of experimental studies (reviewed e.g. by *Godman* 1957 *Field* 1960 *Allbrook et al.* 1966 *Betz et al.* 1966) in which the main interest has been focused on the effect of the satellite cell on the regeneration of muscle fibers and myogenesis (*Carlson* 1970 *Mair* 1970 *Reznik* 1970 *Alon* 1970 *Walker* 1972). In addition certain other problems such as the effect of denervation (*Saunders & Sissons* 1953 local irradiation (*Reznik* 1967) or anabolic and catabolic steroids (*Sloper & Pegrum* 1967 on repair and regeneration in mechanical muscle injuries, have been investigated.

The purpose of our studies is to compare the effect of mobilization and immobilization on the healing of calf muscle injury in the rat. In this type of study it is of the utmost importance that a constant injury can be induced, i.e. the anatomical location as well as the dimensions of the injuries induced must remain constantly within certain sufficiently narrow limits. A method in which no skin wound is needed is preferable, because this may complicate the repair of the injured muscle. The methods used earlier for induction of crushing muscle injuries (*Fishback &*

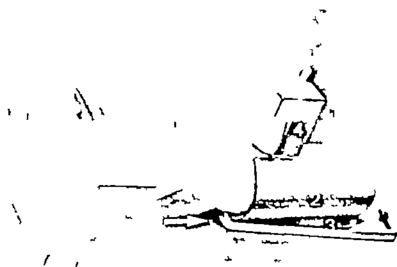

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Fig. 1. The constant trauma inducer is constructed of structural steel and consists mainly of two parts. The hammer mechanism (A in Fig. 1 a) and the test bed assembly (B in Fig. 1 a) which are fixed to each other by means of a bracket mount (1 in Fig. 1 b).

The test bed assembly consists of a hollow cylinder (2) welded on a bracket (3) hinged at one end (→) and allows angular motion in both directions. On top of the hinged end of the hollow cylinder there is a notch bracket (4) with a projection block which has an oval hole with a rubber plug (5). For angular adjustment there are two socket head cap screws (6) which lock the cylinder bracket support in place.

The hammer mechanism contains the coil spring in the cylinder (7) and the push rod with wedge hammer (8) at the end. The hammer mechanism can be locked to the desired height by a hex-bolt (9). The hammer spring tension can be adjusted by displacing the cylinder of the coil spring forward or backward with respect to the support bracket, which is done by turning the lock hex-nuts (10) clockwise or counterclockwise. The final position of the wedge hammer after releasing can be adjusted by turning the wedge clockwise or counter clockwise and locking the wedge hex-nut (11). Above the hammer assembly is the trigger for hammer release (⌂) mounted on the hammer support bracket. On the push rod opposite the hammer wedge there is a setting knob (12) used for gripping the hammer when loading.

Specifications

Hammer:

– mass	90 g
– displacement	34 mm (aver.)
– energy dissipation	4.5 10^6 dyne cm (aver.)

Wedge

– angle	45
– leading edge	round diameter 1.5
– max. thickness	8 mm
– width	20 mm

Spring

– mass	4.1 g
– outer diameter	9.0 mm
– spring constant	7.8 10 dyne/cm
– unload height	94 mm

Spring wire

diameter	1.0 mm
material	stainless steel

Fishback 1932, *LeGros Clark* 1946, *Allbrook et al.* 1966, *Sloper* & *Pegrum* 1967) do not fulfil these criteria.

In this report we describe a new traumatization procedure for inducing a standard crushing injury together with two series of experiments used in testing the method.

MATERIAL AND METHODS

1 Topography of the Injury

Thirty female Wistar rats varying in age from 11 to 13 months and weighing from 194 to 254 g (218.5) were used in this study. All animals were traumatized in both hind legs. They received commercial laboratory animal chow and water *ad libitum* throughout the duration of the study. The animals were individually caged in stainless steel cages with wiremesh bottoms.

The traumatization of the animals was performed in the trauma inducer which is described in detail in Fig. 1. The rat, anaesthetized with ether, was set in a supine position in the hollow cylinder of the test bed assembly. The foot was drawn through the hole and fixed with a bulldog clamp so that the top of the calcaneus bone was positioned just at the level of the lower end of the hole. The foot was further fixed by a rubber plug in the hole against the wall of the notch bracket. The thigh in the notch was fixed to the bracket by a thin rubber band.

The objective of the method was to induce an injury of the calf muscles situated abov the muscle-tendon junction and beneath the point where the hamstrings cross the calf muscles. In every case, it was ascertained that the blow on the leg hit the middle of the calf muscle mass. It was possible to achiev this by rotating the cylinder and caref lly palpating the leg against the hammer. Thereafter the cylinder was fixed, the springhammer drawn up and the blow delivered. A suitable force for our purpose was found in preliminary experiments using varying tensions on the spring.

The animals were killed two days after the injury by a blow on the neck, both hind legs were cut about twenty fiv millimeters above the knee, the skin was removed and the legs fixed in 10 per cent phosphate buffered neutral formalin. Seven days after fixation, the hamstring muscles were removed from the legs and the injured area studied macroscopically with the aid of a magnifying glass. The haemorrhagic area was regarded as representing the injury. A good correlation between this interpretation and actual muscle rupture was observed in the histological examination. The following macroscopic observations and measurements indicated in Fig. 2, were made of the surface of the gastrocnemius muscle.

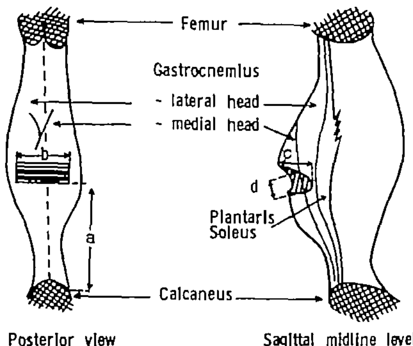


Fig 2 Schematic presentation of the location of muscle injury in the rat calf. The figure represents distances concerning the wound and its location the distance between the calcaneus and the wound (a) the extent of the wound on the surface of the gastrocnemius muscle (b) the depth of the injured area in the sagittal midline (c) and the width of the wound at the level of the fascia between the medial and lateral head of gastrocnemius muscle (d) The sagittal midline connects the top of the calcaneus to the point between the tibial condyli.

1 The course (direction) of the muscle wound, whether transverse or oblique.
2. The distance of the muscle injury from the top of the calcaneus and the breadth of haemorrhagic area were measured.

The calf was then split with a surgical knife along a line connecting the top of the calcaneus the midpoint between the tibial condyli (Fig.) The following observations and measurements were then made in the sagittal plane thus exposed:

1 The depth of the wound was measured and also estimated with regard to different parts of calf muscles in the sagittal plane where the medial head of the gastrocnemius is most superficially situated partly on the lateral head. A little deeper between both heads of the gastrocnemius is located the plantaris muscle with the soleus immediately on the lateral side (Greene 1959) The injured area was studied histologically at the sagittal midline level. The tissue sections were stained by haematoxylin-eosin and van Gieson-haematoxylin methods.

All the measurements were made with a nibble compass, with a degree of accuracy of 0 1 mm

2. *Breaking Strength of the Traumatized Muscle*

Fifteen female Wistar rats were used to study the breaking strength of traumatized muscle (e g the stress by which the muscle is ruptured, Kulund 1971) The weight of the animals ranged from 248 to 283 g (av 267 1) and the age from 5 to 7 months. A control group of five rats matched by age and weight (range from 258 to 287 g, av 271.6) were used. The method of traumatization, breaking and watering were the same as mentioned for the previous study

The animals were killed immediately after traumatization. Both hind legs were cut off as before, the muscles of the thigh were dissected away and the gastrocnemius muscle was removed carefully from the other parts of the triceps surae muscle. The breaking strength was measured with a testing machine which was a modification of that described by *Barfr d* (1971) by fixing femur and foot tightly to the machine. Thereafter the leg was cut above the ankle so that the gastrocnemius muscle was the only connection between the knee and foot. The elongation of the muscle was performed at a constant speed of 0.25 mm/sec and the load stretching was measured. The load measuring system was calibrated each day before and after a series of experiments.

The t-test was used for testing the significance of results.

RESULTS

1 *Topography of the Injury*

The direction of the muscle wound was transversal in 50 out of 60 cases (83 per cent) In all but one of the 10 other cases it deviated only slightly (less than 20) from the transversal direction.

Results of measurements of the distance of the wound from the calcaneus, and of the extent, depth and width of the injured area are given in Table 1

In all cases the rupture penetrated the medial head of the gastrocnemius muscle In two of the 60 cases it extended through the whole gastrocnemius muscle in the plantaris and soleus muscle, but never reached the deep calf muscles (flexor hallucis longus or the flexor digitorum longus muscles) On the average the bottom of the rupture was a little deeper than the middle of the lateral head of the gastrocnemius muscle.

2. *Breaking Strength of the Injured Muscle*

In traumatized muscles the separation (breaking) under traction always took place at the point of the injury while in the control animals the failure site was situated in the muscle belly but never at the tendon part or at the attachment to the femur and to the calcaneus. The results from the maximum load measurements of the breaking strength are presented in Table 2. There were no significant differences between the right and left leg in the same animal in either group, but the difference in breaking strength between traumatized and untraumatized muscles was very highly significant ($p<0.001$) (Table 2)

TABLE 1 *Result from Topographical Measurement of the Muscle Wounds. Number of Legs Measured Was 60 (Means ± SEM)*

Distance	mean mm	SEM mm
wound-calcaneus (a)	19.82 ±	0.17
breadth (b)	6.33 ±	0.11
depth ( )	4.89 ±	0.13
width (d)	2.82 ±	0.03

The letters in brackets refer to the distance indicated in Fig 2.

TABLE 2. *Maximum Load (kp) Measurements of Breaking Strength in Traumatized and Control Muscles (Means ± SEM)*

	Traumatized ($n = 15$ rats)	Control ($n = 5$ rats)	
Left legs	2.59 ± 0.11	3.48 ± 0.07	
Right legs	2.34 ± 0.10	3.27 ± 0.10	
Both legs	2.36 ± 0.06	3.35 ± 0.07	$p<0.001$
	NS	NS	

NS = not significant.

DISCUSSION

Three facts which favour a method in which the induction of an experimental muscle injury is caused without tearing the skin are

1 The risk of infection is eliminated which is of special importance under experimental conditions where the injured leg is immobilized by a plaster cast.
2 The repair of the skin wound, which may complicate the healing process in the muscle injury can be avoided.
3 In athletics, muscle ruptures are not as a rule combined with skin tears.

For these reasons, we have decided to adopt a traumatization method in which no skin tear is caused. When this kind of traumatization is used, however the induced muscle injury cannot be checked visually therefore the method must be reliable enough to bring about a constant degree of trauma in the muscles studied. We were able to induce a standard crushing injury in the rat gastrocnemius muscle in the midline of the calf (Fig. 2) The rupture in each case totally pene

trated the superficial, medial head of the gastrocnemius muscle, whereas the deeper lateral head was only partially injured and the trauma was always situated in the muscle belly of the gastrocnemius below the insertion of the hamstring muscles. Although some variations were observed in the dimensions measured, the standard error of mean values (Table 1) indicate that the injury can be regarded as adequately constant. This also becomes evident even if no significant differences were observed when the breaking strength of the right and left sides of the same animal were compared (Table 2)

Theoretically it should be possible to adjust the force of the hammer-blow so that the penetration of the injury to the deeper located lateral head of the gastrocnemius muscle is kept at a minimum and the plantaris and soleus muscle under it remain relatively undamaged. From our preliminary experiments we concluded, however that the blow of the hammer should be "strong enough" in order to exclude the interference of several disturbing factors (due e.g. to individual anatomical variations in the laboratory animals, reflectory muscle contractions during traumatization and variations in the depth of anaesthesia)

In preliminary experiments we also induced a tetanic muscle contraction with the aid of electrical stimulation during traumatization. Because of the slightly asymmetrical contractions, the wounds thus inflicted caused a variation in the location of the injury some being outside the midline where the samples for histological and histochemical studies were taken. This, of course, reduced the accuracy of the method, since the location of the injury could not be macroscopically established a few weeks after the trauma and even the microscopical identification of the injured area was more difficult.

An important point for investigation in subsequent studies is to get the animals to run on all four legs on the treadmill already on the day after traumatization. The partial rupture of the gastrocnemius muscle induced by our method is suitable for this purpose.

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BRIEF REPORT

THE EFFECT OF PORTOCAVAL ANASTOMOSIS ON THE PERMEABILITY TO HORSERADISH PEROXIDASE OF CEREBRAL VESSELS OF THE RAT

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Laursen, H., Schrøder H. & Westergaard, E. The effect of portocaval anastomosis on the permeability to horseradish peroxidase of cerebral vessels of the rat. Acta path. microbiol. scand. Sect. A, 83 266–268, 1975

The blood-brain barrier to horseradish peroxidase (HRP) was tested in rats with hepatic *encephalopathy induced by portocaval anastomosis. An increased number of vesicles containing* HRP was seen in endothelial cells. Basement membranes of some vessels and the adjacent neuropil were filled with HRP Astrocytes showed swollen end-feet. The cytoplasm of astrocytes adjacent to HRP containing basement membranes was evenly filled with HRP This phenomenon could be caused either by cellular hyperactivity or by leaking membranes due to cell damage.

Key words Portocaval anastomosis hepatic encephalopathy; blood-brain barrier horseradish peroxidase vesicular transport astrocyte.

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Morphological changes in astrocytes have been described in patients with hepatic encephalopathy (1 2, 5 11 14) and also in animals with experimentally induced hepatic encephalopathy (4 7 8, 9 16, 17 21) The close relation between the astrocytes and cerebral vessels raises the question of whether or not the blood-brain barrier (BBB) is affected by the above-mentioned conditions. Increased permeability to cyanol and neutral red has been observed in dogs with encephalopathy induced by portocaval anastomosis (PCA) (13) In another investigation the permeability to neutral red, bromide, and potassium iodide was normal (10) In rats with hepatic encephalopathy induced by CCl the permeability was enhanced to silver nitrate but not to Evans blue (15)

The BBB to horseradish peroxidase (HRP) under normal conditions has been the subject of several examinations. Tight junctions seal the endothelial cells, preventing intercellular passage (18, 6) Recently a minor vesicular transport of HRP in parts of arterioles was demonstrated (20). Furthermore, a very slight transfer by vesicles has been observed in portions of venules and capillaries (19) Increased permeability to HRP was found under various pathological conditions, for example hypertension (3)

The purpose of this preliminary study is to investigate whether or not hepatic encephalopathy induced by PCA influences the BBB to HRP

Material and Methods

PCA was carried out on six adult, male rats in ether anaesthesia (12) The animals were fed ad libitum on an ordinary commercial diet. In Nembutal anaesthesia ten days after the operation 200 mg HRP (Sigma, type II) was injected intravenously in the course of two mins. Three mins later the rats were fixed by vascular perfusion with a mixture of paraformaldehyde and glutaraldehyde in a cacodylate buffer The brains were removed, cut into one-half mm thick frontal slices, and in-

cubated for peroxidase activity. Small pieces of tissue from the cortex cerebri and cortex cerebelli, basal ganglia and pons were treated with osmium, impregnated with uranyl acetate, dehydrated, and embedded in Spurr plastic for electron-microscopic examination. Six other rats were sham-operated. The abdomen was opened, the coronary venous tributary to the portal vein was ligated, and the portal vein and the inferior vena cava clamped for 12 min *corresponding to the duration of the operation* on the rats with PCA. The further treatment was as for rats with PCA.

Results

In the rats with PCA the cerebral vessels were studied. The endothelial cells, especially of the arterioles, exhibited a greater number of vesicles filled with HRP than normal. The intercellular *spaces of the endothelial cells* were devoid of HRP between the first luminal and first abluminal tight junctions. The basement membranes of several arterioles contained HRP and the extracellular spaces of the neuropil adjacent to these vessels were filled with HRP.

The end-feet of astrocytes facing the walls of the *vessels* were swollen. *If* the extracellular space of the neuropil contained HRP, the cytoplasm of the foot processes could show evenly distributed HRP and tracer could also be demonstrated around the nucleus of the *astrocyte* and in the processes without relation to vessel walls (Fig. 1). In astrocytes there was no HRP in vesicles and other *membrane-limited* structures.

Neurons could exhibit vesicles containing HRP but the number was not increased compared with the controls. Mitochondrial morphology was normal not only in neurons and astrocytes, but also in endothelial cells.

In sham-operated control rats there were none of the pathological *findings* in rats with PCA described above.

Comments

I[n] hepatic encephalopathy induced by PCA we have observed *an increase in* the number of vesicles containing HRP in endothelial cells and a large amount of tracer in the basement membranes of the vessel *walls* and in the extracellular spaces of



Fig. 1. HRP-reaction product is observed in the basement membranes (BM) in the wall of an arteriole. L: lumen, SM: smooth muscle cell. Furthermore reaction product is distributed in the cytoplasm of an *adjacent* astrocyte extending into the neuropil (arrow). N: nucleus of the astrocyte. × 5000

the neighbouring neuropil. Furthermore, there was no HRP between endothelial cells and since, these cells were intact, it is reasonable to assume that there had been increased vesicular transport across the endothelium.

An explanation of the diffuse uptake of HRP into the cytoplasm of astrocytes with swollen end feet but with mitochondria seeming ultrastructurally normal may be that these cells are hyperactive in taking up substances of high molecular weight. Another explanation could be leaking membranes, caused by cell damage in this stage of PCA-induced hepatic encephalopathy

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Acta path. microbiol. scand. Sect. A, 83: 269–282, 1975

HEALING OF A CRUSH INJURY IN RAT STRIATED MUSCLE

2. *A Histological Study of the Effect of Early Mobilization and Immobilization on the Repair Processes*

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Järvinen, M. Healing of a crush injury in rat striated muscle. 2. A histological study of the effect of early mobilization and immobilization on the repair processes. Acta path. microbiol. scand. Sect. A, 83: 269–282, 1975.

The effects of both early mobilization and immobilization on the healing of a crush injury in the rat gastrocnemius muscle were studied histologically. A reproducible injury was induced on the left calf of 242 rats. The histological observations were made 2 to 42 days after the injury and the intensity of the changes was estimated semiquantitatively by scoring. During the first week, the histological changes were especially dependent on the post-traumatization treatment. The inflammatory reaction and haematoma formation were more pronounced in the mobilized muscles and the amount of necrotic and degenerated muscle tissue was more in the immobilized muscles. The disappearance of the haematoma and of the inflammatory cells occurred more rapidly with mobilization. Scar formation was more pronounced in the mobilized injuries. Immobilization for the first 2–5 days after the trauma was followed by a slight delay in the maturation and contraction of the fibrous scar. Muscle regeneration was more extensive and occurred more rapidly in the mobilized muscles. The penetration of regenerated muscle fibres through the scar was more pronounced in the immobilized muscles, but the orientation of the new fibres was more parallel with the original muscle fibres after mobilization. The histological differences between the treatment groups diminished markedly towards the end of the observation period.

Key words: Crush injury; experimental; striated muscle; healing; histology; treatment; mobilization; immobilization.

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The repair of striated muscle after acute trauma, such as a rupture or contusion, occurs simultaneously by the regeneration of disrupted muscle and formation of a connective tissue scar (*Miller* 1934, *Field* 1960, *Allbrook et al.* 1966, *Betz et al.* 1966, *Gallucci et al.* 1966, *Carlson* 1968).

Due to the large frequency of muscle injuries in athletes, their treatment in sport medical practice is of importance (*Corrigan* 1965, *Millar et al.* 1967, *Bass* 1969, *Barry* 1969, *Ryan* 1969). Previously immobilization was the only method used in the treatment of muscle injuries (*Hess* 1956, *Thorndike* 1956, *Hinrichsen* 1963) and troublesome complications e.g. reruptures and loss of muscle extensibility and strength often followed. Mainly for this reason, early mobilization of injured limbs has, during the last two

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decades, come to be regarded as the method of choice in the treatment of injured muscles (*Woodard* 1954 *George* 1963 *Corrigan* 1965 *Miller et al.* 1967 *Bass* 1969 *Ryan* 1969)

Scattered experimental studies indicate that early activity provides a suitable environment for soft tissue repair and especially for muscle regeneration after trauma (*Allbrook et al.* 1966) Although the effects of various external factors, e.g denervation (*Saunders & Sissons* 1953) irradiation (*Reznik* 1970) catabolic and anabolic steroids (*Sloper & Pegrum* 1967) and agents capable of blocking the maturation of collagen (*Gallucci et al* 1966) upon the repair of striated muscle injury has been previously studied, prior to our preliminary study (*Kvist et al* 1974) no attention has been focused on the healing of injured muscle in various physiological conditions.

In clinical practice, on the other hand, the length of the rest period following trauma before mobilization is commenced is a subject of dispute today and varies in length from one to five days. Therefore in this study where the main focus was to make a histological comparison of the effect of mobilization and immobilization on the healing of crushed muscle combination of these treatments were employed in schedules similar to those in clinical use.

MATERIAL AND METHODS

Experimental A imals

Mal albino rats of the Wistar strain (total number 242) were used in this study At the time of traumatization their average age was 20 weeks (range 12–51 weeks) and their body weight aried from 234 g to 363 g (mean 288 ± 2 g ± SEM) The animals were housed in wire mesh cages and received laboratory chow (Hankkija, Finland) and water *ad libitum*.

Animals younger than 12 weeks were not used because in the muscle fibres of young animals there is a large amount of satellite cells the number of which decreases to a constant level during the first three postnatal months (*Allbrook* 1973) As regenerative capacity is closely related to the satellite cells, a relatively stable regenerative capacity of muscle fibres was thus obtained.

Traumatization of the Animals

The animals were first taught to run on the treadmill Immediately before traumatization they were weighed and divided into five groups matched according to body weight A [illegible] transverse contusion injury using a blunt spring loaded hammer was induced on the left calf of each animal under ether anesthesia. The force of the spring was adjusted so that the gastrocnemius muscle was partially crushed without tearing the skin. This traumatization procedure has been described in detail (*Järvinen & Sorvari* 1975) The spring constant remained unchanged during the experiments. The time of traumatization is designated day 0 and the consecutive days are called days 1 2 etc.

Post Tra matization T eatment of the Animals

Group MO The rats were exercised each day beginning on day 1 in a motor-driven treadmill with an inclination of 15 at a speed of 40 cm/sec The training time on the first day consisted of two 20 minutes periods, about 60 min apart from each other on day 2 of two periods of 30 minutes, and thereafter of 60 minutes once daily The same mobilization program was also used in our preliminary study (*Kvist et al.* 1974) and is rather similar to one used earlier by *Tipton et al.* (19[illegible] During the experimental period the animals were weighed daily

Group IM1 + MO The animals were treated in a manner similar to that used in Group MO except that immediately after traumatization the injured limb was immobilized with a [illegible] plaster cast for one day

Group IM2 + MO The rats were treated with a plaster cast around the contused leg for two days after traumatization and mobilized thereafter as described above.

Group IM5 + MO The treatment of these animals was the same as that used in Group IM2 + MO except that the immobilization period after trauma was five days

Group IM The rats were treated by immobilization alone. Immediately after trauma the contused leg was immobilized with a softened plaster cast (Cellona® Lohman KG Fahr/Rhein, Germany) to about 1 cm abov the knee. Casts were [illegible] fied daily and replaced and repaired whenever necessary The dry weight of each cast was about 15 g. During the first day of immobilization, the body of the rat was covered with a cylindrically shaped piece of plastic-covered wire net to prevent the animal from eating the plaster cast. After one day the cylinder was removed.

In Groups MO and IM the animals were killed on days 2, 5 7 14 21 and 42, in Group IM1 + MO and IM2 + MO on days 7 14 21 and 4[illegible] and in Group IM5 + MO on days 14 21 and 4[illegible]

TABLE 1 *The Number of the Rats*

Days after injury	2	5	7	14	21	42	Total
Group MO treated by mobilization alone	11	10	11	11	11	11	65
Group IM1 + MO treated by mobilization after immobilization for one day			10	10	10	9	39
Group IM2 + MO treated by mobilization after immobilization for two days			10	11	9	11	41
Group IM5 + MO treated by mobilization after immobilization for five days				11	10	11	32
Group IM treated by immobilization alone	11	10	11	11	11	11	65
	22	20	42	54	51	53	242

by a blow on the neck. The rats which were killed on day 42 were treated during the first three weeks only and kept thereafter free in cages without immobilization or treadmill mobilization. The number of animals in the different groups is presented in Table 1.

Preparation of the Calf Muscles

After killing, the animals were weighed and both hind legs were severed about 25 mm above the knee, the skin was removed and the legs were fixed in 10 per cent phosphate buffered neutral formalin. After fixation for 1–2 weeks the hamstring muscles were removed from the legs and the injured area was studied with a magnifying glass. The following measurements of the surface of the gastrocnemius muscle were made:

the distance of the injury from the top of the calcaneus (to document the constancy of the location of the injury in the calf)
the breadth of the injured area (haematoma or scar)
the largest circumference of the leg
the volume of the lower leg

Measurement of the Extent of the Muscle Injury

The calf was then split with a surgical knife along a line connecting the top of the calcaneus with the midpoint between the tibial condyles and the following further observations were made in the sagittal plane thus obtained:

the depth of the haemorrhagic area or scar was measured
the width of the injury was measured along the fascia between the medial and lateral heads of the gastrocnemius muscle. In the sagittal sections, the medial head is situated most superficially, partly overlying the lateral head.

After two days of additional fixation in formalin, the medial half of the calf muscles was dehydrated in graded alcohols, cleared in xylene and embedded in a tissue-embedding medium containing paraffin and plastic polymers (Fibrowax, supplied by Bethlehem Trading, Göteborg, Sweden). From the midline level of the muscle blocks, sections of 6 μm thickness were taken for histological study. From our preliminary study (*Kvist et al.* 1974) where the gastrocnemius muscle was cut in serial sagittal sections, it had become evident that the most representative level for histological study was at the middle of the injured area. The sections were stained by the Haematoxylin-eosin and van Gieson-haematoxylin methods. In addition, Gomori's staining method (*Romeis* 1948) was used for reticulin fibres and Verhoeff's staining method (*Mallory* 1944) for elastic fibres.

Microscopical observations and semiquantitative estimations of the following histological characteristics were made twice without knowledge of the group to which each of the specimens belonged:

the depth and width of the injured area and the breadth of the transitional zone (*Carlson* 1970) between the necrotic and totally destroyed tissue or "pure" connective scar and the uninjured tissue were estimated both to ascertain the constancy of the trauma and to investigate the amplitude of histological changes following the injury after different methods of treatment. The depth was estimated with respect to the fascial plane between the calf muscles. The width of the injured area and breadth of the transitional zone were estimated with respect to the high power (40 ×) field of the microscope (about 5.5 mm in diameter).

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– the relative amounts of polymorphonuclear leucocytes, macrophages, other mononuclear cells, fibroblasts, collagen, reticulin and elastic fibres were semiquantitatively scored using numbers from 3 (abundant) to 1 (rare) and 0 (none). The size of the necrotic area was estimated from 2 to 0. Hyaline, vacuolar granular and discoid types of degeneration in striated muscle cells were identified according to *Allbrook et al.* (1966) and for these a scoring from 2 (abundant) to 0 (none) was adopted.

The following morphological features presented by *Reznik* (1970) and *Carlson* (1968) were used as *criteria of muscle regeneration: proliferation of* myoblasts, formation of myotubes and the development of new myofibres. The extensiveness of the regenerative process was scored from 3 (regenerative potential found in practically all stumps of crushed fibres) to 0 (no regenerative activity in the area of trauma).

The results of the measurements and estimations using this scoring system were analysed statistically the significance of the differences between two groups being evaluated by Student's t-test and between more than two groups, by variance analysis.

RESULTS

Measurements and Gross Observations

Body weight. The mean values of changes in body weight during the observation period are presented in Fig 1. During "active" treatment period of 21 days, the rats in all groups lost weight (most in Group IM on day 21 10.7 per cent). The overall gain in weight of rats studied on day 42 varied from 3.2 per cent (Group IM) to 18.6 per cent (Group IM5 + MO) compared with body weight on day 0.

Leg circumference. As indicated in Fig 2, the maximum circumference of the injured legs immobilized with a plaster cast decreased significantly during immobilization (mean values on days 2 and 21 after injury were 47.2 ± 1.0 mm and 42.5 ± 1.1 mm, $p < 0.01$) and the differences between the injured and the contralateral nontraumatized calfs were highly significant on days 14 and 21 ($p < 0.001$). In the rats treated by mobilization there were no differences between the circumference of the legs during the entire observation period (Fig 2). From day 21 to 42 there was an increase in the circumference of both legs in all groups except the nontraumatized control legs of rats in Group IM which remained unchanged. The measurements of calf circumference paralleled the volume measurements, indicating atrophy of muscular tissue during immobilization followed by increase of muscle mass after removal of the plaster cast. In mobilized rats the hypertrophy of the calf muscles was ex-

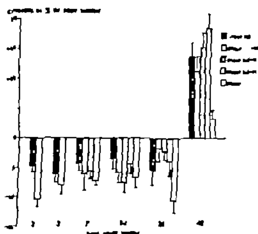


Fig 1. Changes of body weight per cent of weight at traumatization. In all figures each value represents the mean ± standard error of the mean of 9 to 11 rats.

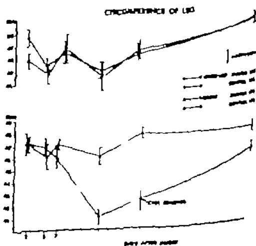


Fig 2. Largest circumference of each hind leg the rats treated by mobilization (Group MO) a immobilization (Group IM). The measurements were made after removal of the skin.

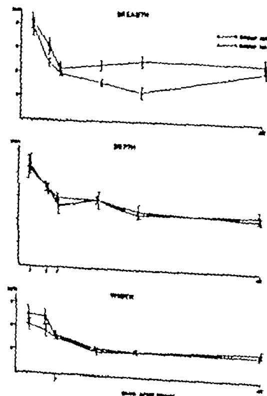


Fig 3 Results of the topographical measurements of the injured area in mobilized (Group MO) and immobilized (Group IM) rats

dent after day 14 (Fig 2) Macroscopically the marked muscular atrophy in the immobilized legs was evident on day 7 and increased up to day 21 In Groups IM1 + MO to IM5 + MO the circumference measurements were similar to those in Group MO and no atrophy was observed. Thus, it became evident that both mobilization and immobilization were effective, leading to hypertrophy or atrophy respectively of muscle tissue

Measurement of the injured area The measurements of the injured area are presented in Fig 3 There was a conspicuous decrease in both the breadth and width during the first week, but later a decrease only in the breadth of the injuries in Group IM was observed The results of these measurements in another group did not differ from those in Group MO

Gross observations Two days post traumatization, the retraction cap on the surface of the injured muscle was filled with a haematoma, larger in Group MO than Group IM The disappearance of the haematoma during the first week was followed by the formation of a scar This diminished in size toward the end of the observation period (day 42) at which time there was only a small pale notch on the surface of the gastrocnemius.

Microscopical Observations

Day 2 The injured area extended through the medial head of the gastrocnemius muscle in all cases and reached the plantaris in two cases, but generally the rupture extended to a point near the dense fascia under the gastrocnemius muscle. The width of the in-

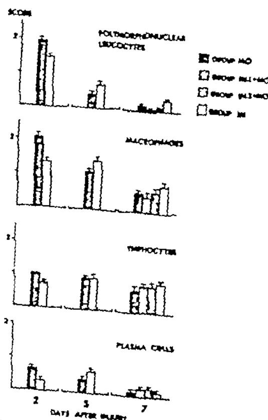


Fig 4 Effect of the various mobilization schedules on the intensity of the inflammatory cell reaction during the first week after injury



Fig 5 Effect of the various mobilization schedules on the number of myotubes/injured area. One section of each rat is connected.

Fig 6 Muscle injury 2 days after traumatization, Group IM. A large area with necrotic and degenerated muscle fibres with a few inflammatory cells in the transitional zone of the injury is seen. van Gieson-haematoxylin, 120 ×

Fig 7 Muscle injury 2 days after traumatization, Group MO. The large haematoma in the middle of the crushed area (to the left in the figure) and the inflammatory cells in the transitional zone are more numerous than in Fig. 6. van Gieson-haematoxylin, 1*0 ×

Fig 8 Part of the transitional zone of the injured area, 2 days after trauma, Group IM. Some polymorphonuclear leucocytes and macrophages, but no fibroblasts between the injured muscle fibres. Hyaline and granular degeneration. van Gieson-haematoxylin, 300 ×

Fig 9 Part of the transitional zone of the injured area 2 days old, Group MO. Abundant inflammatory cells and fibroblasts between the injured muscle fibres and activated myoblasts at the ends of crushed muscle fibres. van Gieson-haematoxylin, 300 ×

jured area nearly covered the dry high power field (40 ×) of the microscope. The mass of traumatized muscle was thus rather constant.

The innermost zone of the traumatized area contained crushed, totally disruptured, necrotic muscle fibres. Despite necrosis in the affected fibres, degenerative changes of the hyaline, granular and vacuolar types were conspicuous more prominent in Group IM than in Group MO (mean score values 2.5 and 2.0 respectively $p < 0.05$) (Fig. 6 and 7). Among the disintegrating, crushed fibres in the innermost zone there were large amounts of erythrocytes and fibrin clots. The haematoma in the middle of the traumatized area was prominent significantly more often in Group MO than Group IM ($p < 0.001$).

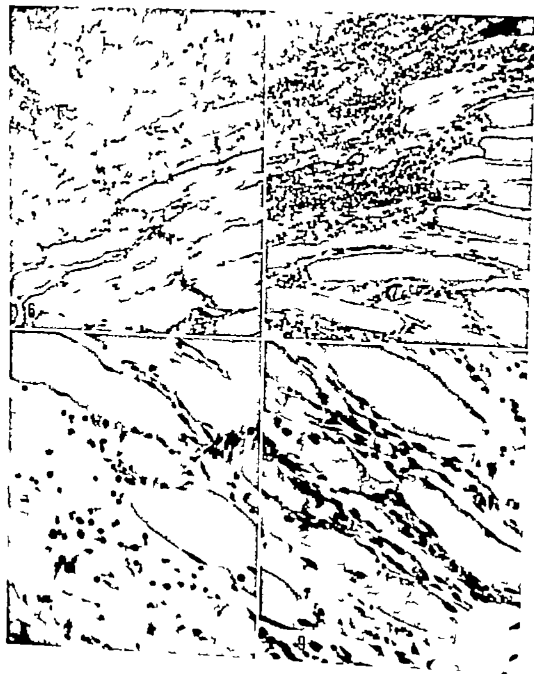
Inflammatory cell infiltration was most prominent in the transitional zone around the stumps of surviving muscle fibres. In particular polymorphonuclear leucocytes and macrophages were more abundant in Group MO than in Group IM (mean values of scores 2.0 2.1 and 1.5 1.4 respectively $p < 0.01$) (Fig 4 6 7 8 and 9). The apparent myoblasts with large spherical and ellipsoid shaped nuclei, often with two nucleoli, were seen inside the sarcolemmal sheats near the stumps of muscle fibres, a little more frequently in Group MO than in Group IM. However no myotubes were found at the early phase (Fig 6 7 8 and 9).

In mobilized muscles (Group MO) there were numerous spindle-shaped cells, regarded as fibroblasts among the inflammatory cells (Fig 9). By contrast, practically no fibroblasts were found in the immobilized muscles (Group IM $p < 0.001$) (Fig 8).

No reticulin fibres were identified around the crushed muscle fibres and no signs of the production of new fibres were yet present.

Day 5 The degenerative changes in the muscle fibres were less than on day 2 in both groups and especially the granular type was almost absent in Group MO. The amount of polymorphonuclear leucocytes was considerably less than on day 2, more so in Group MO than in Group IM and the macrophages were more numerous in Group IM (mean values of scores 1.5 and 1.1 $p < 0.05$) (Fig 4 10, 11).

The haematoma in the muscles of rats in



Group MO was more prominent than that in the immobilized muscles (Groups IM).

Muscle regeneration was distinctly more intense in Group MO than in Group IM. In the mobilized muscles, numerous myotubes (mean 32 myotubes/microscopical section) were found in the injured area with numerous myoblasts (Fig. 5 and 11) while in the immobilized muscles no or only a few myotubes (mean 8 myotubes/microscopical section) and only moderate myoblasts were observed (Fig. 5 and 10).

The fibroblasts in the traumatized area were more numerous than on day 2 in both groups and abundant reticulin and moderate amounts of fine young collagen fibres had appeared in the granulation tissue in animals in Group MO but only few connective tissue fibres were found in those in Group IM ($p < 0.001$)

Day 7 In Groups MO to IM2 + MO the muscle degeneration was minimal and the granular type was totally absent (mean scores 1.1 1.0 and 1.1 respectively) In contrast, moderate degeneration was evident in Group IM (mean score 1.5 $p < 0.05$ between Groups MO and IM) (Fig 12 and 13) A haematoma was still present in about half of the cases in each group

The inflammatory reaction was much less than on day 5 polymorphonuclear leucocytes had nearly totally disappeared and the number of lymphocytes and macrophages was slight (Fig 4) There were no intergroup differences in the inflammatory reaction.

The regeneration of muscular tissue was pronounced, represented by abundant myotubes penetrating through the granulation tissue with some young collagen fibres in the central zone of the injury The myotube formation reached its maximum at this time and the differences in the amount of myotubes in animals in the various groups had *nearly disappeared* (*Fig 5*) *An increased* amount of collagen fibres was observed in Groups MO to IM2 + MO but only few was found in Group IM Abundant reticulin fibres were observed around the fibroblasts in the granulation tissue and also in the endomysium of the new myotubes. No elastic fibres were present in the young connective tissue. The orientation of collagen fibres and myotubes was parallel with the noninjured muscle fibres in Group MO to IM2 + MO In *Group IM their orientation was more* interlacking ($p < 0.001$ between Groups MO and IM) (Fig 12 and 13)

Day 14 The area with signs of injury was markedly diminished in all groups in comparison with day 7 The depth reached to the lateral head of the gastrocnemius muscle and the width averaged about half of the dry high power field (40 ×)

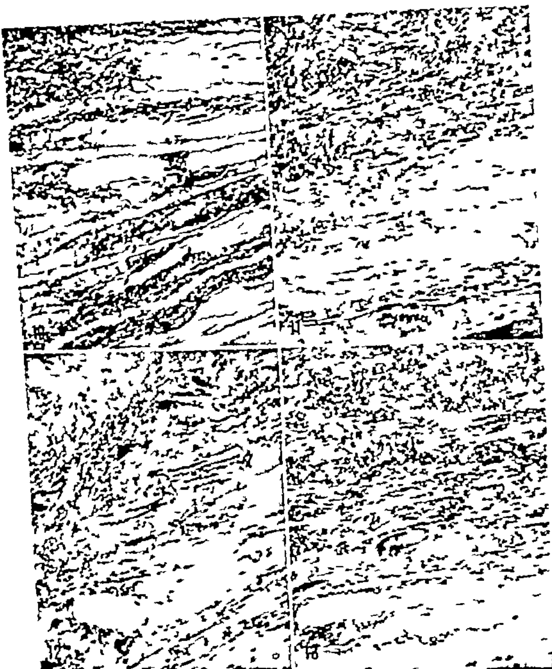
Inflammatory cells and degenerative changes in the muscle fibres were absent from all injuries. The number of myotubes was less than on day 7 the greatest reduction occurring in the mobilized groups (*Fig. 5*) The young muscle fibres, thicker in diameter than the myotubes, were more numerous and the nuclei had already migrated to the periphery of the fibres. Some muscle giant cells were observed at the ends of regenerated muscle stumps, terminating in the connective tissue scar at the middle of the injury *the number* being largest in Group IM. In the mobilized groups, the collagen increased in comparison with that on *day 7 and it had matured.* The size of the scar was more pronounced in Groups IM2 + MO and *IM5* + MO (mean scores 2.4 and 2.3 respectively) being at a minimum in Group *IM* (*mean* score 1.3 ($p < 0.001$ between IM and both IM2 + MO and *IM5 + MO*) *Between* Group MO

Fig 10 Muscle injury 5 days after trauma, Group IM. Necrotic muscle in the middle of the injured area (at the left of the figure) numerous inflammatory cells, fibroblasts and some fine young connective tissue fibres at the border of the injury No myotubes are found. van Gieson-haematoxylin, 120 ×

Fig 11 Muscle injury 5 days after trauma, Group MO Extensive new connective tissue fibres in the middle area of the trauma (to the left in the figure) and numerous myotubes from the stumps of crushed fibres towards the centre of the injury van Gieson-haematoxylin, 120 ×

Fig 12 Injured area of the muscle 7 days posttraumatization, Group IM. Few inflammatory cells in the transitional zone. New connective tissue fibres in the middle of the injury are not parallel with the old muscle fibres. Numerous myotubes and vacuolar degeneration at the end of old muscle fibres are found in the middle of the figure. Haematoxylin-eosin, 120 ×

Fig 13 Injured area of the muscle 7 days posttraumatization, Group MO Abundant myotubes *among* the *new* connective tissue fibres in the transitional zone are parallel with the old muscle fibres compared with Fig 12. Haematoxylin-eosin, 120 ×



(mean score 1.8) and Groups IM2 + MO as well as IM3 + MO the differences were also significant ($p < 0.01$ and $p < 0.05$ respectively). The amount of reticulin was less than on day 7 but no elastic fibres were found among the scar. The penetration of regenerated muscle fibres through the scar was more prominent in Group IM (mean value of scoring of penetration in Groups MO to IM3 + MO 1.7, 1.6, 1.5, 1.5 and in Group IM 2.2, $p < 0.05$ between MO and IM).

Day 21 The injured area estimated according the histological changes was still smaller than on day 14 but in all cases the depth of the injury reached the lateral head of the gastrocnemius muscle. The repaired tissue was also mature, and the number of myotubes was, especially in Group IM further diminished Young muscle fibres, smaller in diameter than in the original part of muscle, connected the borders of the injury gap

In Group IM the muscle giant cells at the ends of the regenerated muscle fibres were more numerous than on day 14 (Fig 14) In the other groups their amount was greatly decreased. The scar was smaller than on day 14 in all groups of mobilized animals, but larger in Group IM (the mean values of the scores of all the groups varied from 1 6 (MO) to 2 0 (IM5 + MO) $p < 0.05$ between MO and IM5 + MO) Thickening of the endomycium around the new muscle fibres was observed in a wider area in Group MO than in Group IM ($p < 0.05$)

The new muscle fibres were usually oriented parallel to or slightly divergent from the longitudinal axis of the "old" fibres, but in Group IM irregularly interlaced fibres in the middle of the injured area were found more often than in mobilized groups.

The elastic fibres were observed in the scar tissue in nearly all injuries belonging to Groups MO to IM5 + MO but in only one injury in Group IM

Day 42 The injured area was almost completely occupied by new muscle fibres with normal morphology although many of them had cross connections and some of the regenerated fibres in the middle of the injured area were not oriented parallel with the "old" muscle fibres (Fig 16 and 17)

Particularly in the mobilized muscles, the angulation of regenerated fibres close to the scar was pronounced so that their parallel orientation near the scar became more complex (Fig 17)

The fibrous scar was mature and smaller than on day 21 in many injuries it was only a narrow septum transverse to the muscle fibres. No intergroup differences in the size

Fig 14 Muscle injury 21 days after traumatization Group IM. The scar is smaller and the regenerated muscle tissue is mature In the middle of the injury are numerous muscle giant cells van Gieson-haematoxylin, 120 ×

Fig 15 Injured area, 21 days old, Group MO. The amount of connective tissue scar is larger than in Fig 14 Generally the regenerated muscle fibres *are parallel and only a few irregular fibres in the* middle of the injury are seen. Haematoxylin-eosin, 120 ×

Fig 16 Injured muscle 42 days after trauma, Group IM. Regenerated muscle fibres are mature and connective tissue has considerably diminished. Some irregular situated muscle fibres in the middle of the repaired area. van Gieson-haematoxylin, 120 ×

Fig 17 Injured muscle 42 days after trauma, *Group MO* The regenerated muscle fibres are mature and the connective tissue has contracted. Hence, the new muscle fibres in the injured area are angulated cross sections of many regenerated fibres are found. van Gieson-haematoxylin, 120 ×

of the scar were found. Muscle giant cells were fewer than on day 21 even in Group *IM* but distal to the injury in close connection with a dense and large fibrous scar near the fascia under the gastrocnemius muscle there were still some myotubes with degenerated changes.

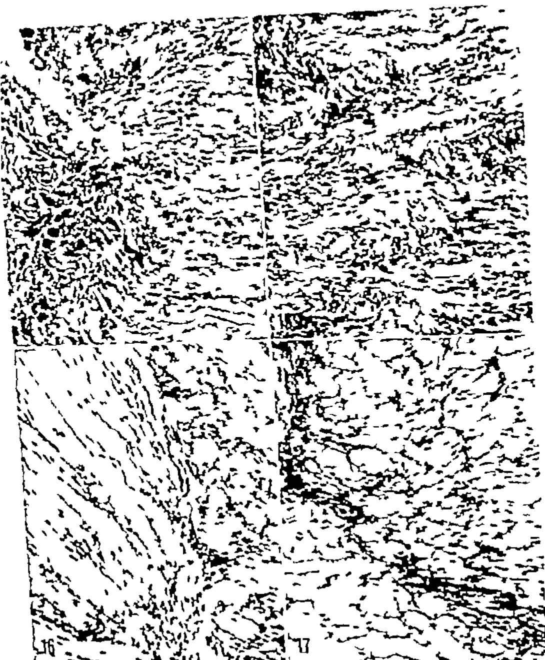
The amount of elastic fibres in the scar was greater on day 42 than day 21 however, their number at this time was generally limited.

The histological differences between the various treatment groups were markedly diminished toward the end of the observation period.

DISCUSSION

Methodological Aspects

In this kind of study it is important that 1) The trauma induced is constant and 2) the treatment, mobilization or immobilization is sufficiently effective. The reproducibility of the injury was tested in an earlier study (*Järvinen & Sorvari*) on the second day after injury (day 2) and was well de-



monstrated in the present study both through the measurements of the injured area and histological observations also on day 2. On days 14 to 42 the traumatized area was observed to extent only slightly under the fascia between the medial and lateral heads of the gastrocnemius muscle and was shallower than on day 2. The explanation of this phenomenon appears to be that the connective tissue sheats of injured muscle fibres at the deeper levels of the injury remained intact. As it is known that the basement membranes

of injured fibres serve as a scaffold for regenerating muscle fibres (*Vracko & Benditt* 1972) the complicating effect of scar formation was avoided. Hence the regeneration was complete after maturation it was impossible to distinguish these fibres from the uninjured muscle.

An injury as that used in this study also allowed mobilization of the rats which used all four legs on the treadmill on the first day following traumatization. The mobilized traumatized leg did not undergo atrophy as compared with the contralateral uninjured leg, indicating the effectiveness of mobilization. This has been discussed in greater detail in a previous study together with some supplementary experimental evidence (*Kvist et al.* 1974).

On the other hand, the marked muscular atrophy in the immobilized leg, as compared to the contralateral leg demonstrated the effectiveness of the immobilization with the softened plaster cast. A plaster cast is the most common method of immobilization in clinical treatment and has also been used in experimental studies (*Cooper* 1972).

Effect of Mobilization and Immobilization on the Healing of Muscle Injury

Although the morphology and histological sequence of events after muscle injury are well-known and have recently been presented and discussed in two comprehensive reviews *Allbrook* (1973) and *Carlson* (1973) rather little is known about the effect of muscular activity or inactivity on the healing of muscle injury. The results of this study show that the post traumatization treatment with mobilization has a different effect on the repair process than immobilization.

The histological differences between injuries treated in the various ways were more pronounced during the first week after the trauma. The haematoma in the middle of the injured area was larger and the inflammatory cells in the transitional zone were more abundant in the mobilized muscles, while the amount of necrotic tissue and the appearance of degenerative changes were more pronounced in the immobilized muscles. The haematoma, inflammatory cells, necrotic tissue and degenerative changes disappeared more rapidly in muscles treated by mobilization. The events of repair, regeneration of muscle tissue and formation of connective tissue scar were more intensive and occurred more rapidly under mobilization treatment. The speed of regeneration was indicated by the appearance of myotubes, and later by their more rapid diminution when they matured to young muscle fibres. The speed and intensity of scar formation was indicated by the early appearance of fibroblasts, reticulin and collagen fibres, and later (day 21) by the appearance of elastic fibres in the injured area. Immobilization for only 2 or 5 days immediately following the trauma was observed to result in delayed contraction and maturation of the fibrous scar on the third week after the injury.

Muscle giant cells, which were more numerous in the immobilized muscles and increased from day 14 to 21 in that group, were regarded as an early atrophic change in regenerated fibres (*Allbrook et al.* 1966). The few atrophic myotubes observed in injuries in animals in all the treatment groups on day 42 were generally situated near the dense fibrous scar distal to the injured area. They remained apparently without connection with the nervous system, because the lack of innervation is followed by a delayed maturation of regenerated muscle fibres after the myotube phase (*Carlson* 1973, *Schick & Jerusalem* 1973).

The observation on mobilized muscles that myotubes and young collagen fibres were oriented parallel with the original muscle fibres may be of clinical importance. *Denny-Brown* (1951) and *Carlson* (1968) observed the same phenomenon in injured muscle maintained at normal muscular tension; the lack of tension, e.g. in denervated muscle is followed by randomly oriented regenerated muscle and connective tissue fibres which are also often more interlacing. Normal muscular tension in healing muscle also increases

the speed of repair as compared with healing in muscles not under tension (*Denny-Brown* 1951)

In the present study mobilization led to results similar to those produced by normal muscular tension and correspondingly immobilization led to results similar to those produced by denervation.

In the mobilized muscles there was an increased angulation away from the longitudinal axis in the regenerated muscle fibres inserting on to the scar. This was due not only to the normal contraction of the scar but was probably also caused by the hypertrophy of the fibres in the mobilized muscles, in a manner similar to the increased angulation near muscle insertions in exercise hypertrophy described by *van Linge* (1962)

In addition to muscular tension, the healing of injured muscle is also dependent on the endocrine status of the animal and particularly on the blood supply and oxygenation of the injured tissue (*Hudgson & Field* 1973) The retarding effect of cortisone and the enhancing effect of methandienone (an anabolic steroid) on the healing of injured muscle were demonstrated by *Sloper & Pegrum* (1967)

In the present study the catabolic state, generally following injury was enchanced by the immobilization favouring muscle atrophy On the contrary mobilization apparently tended to overcome the catabolism produced by the injury and led to an increase of the anabolic state favouring muscle hypertrophy

According to some authors, however the actual histological evidence of progressive regeneration is mainly dependent on the ingrowth of vascularity to the injured area (*Carlson* 1971 *Snow* 1973) *Snow* (1973) using histochemical methods, observed that the speed of muscle regeneration, especially the formation of myotubes was directly related to the level of aerobic metabolism in healing tissue. On the other hand the speed of connective tissue scar formation and especially proliferation of fibroblasts in soft tissue wounds are generally closely connected to the oxygen tension which is directly related to the circulation (*Niinikoski* 1969 *Niinikoski et al* 1972) Hence, both muscle regeneration and scar formation seem to be dependent upon the blood supply to the injured area.

Muscle blood flow is increased in muscle, not only during exercise, but also to a moderate extent following immobilization after a lapse of time during which the muscle undergoes atrophy (*Hudlická* 1973) Hence, the circulatory differences between muscles treated by mobilization or immobilization seem to diminish with time. This may partly explain the disappearance of histological differences between the groups in the late phase of healing.

The various methods of post traumatization treatment employed in this study have clearly differing effects both on the speed of events involved in muscle repair and the structural organization of the reparative tissue elements. Further studies are in progress to clarify the metabolism and circulation in the injured area and the mechanical properties of traumatized muscle

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Acta path. microbiol. scand. Sect. A, 83 283–291 1975

PULMONARY VASCULAR LESIONS IN CHICKENS FOLLOWING INTRAVENOUS INJECTIONS OF DISINTEGRATED CELLS OF *ESCHERICHIA COLI*

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Nordstoga, K. & Fjølstad, M. Pulmonary vascular lesions in chickens following intravenous injections of disintegrated cells of *Escherichia coli*. Acta path. microbiol. scand. Sect. A, 83 283–291 1975

Intravenous injections of disintegrated cells of *Escherichia coli* in chickens were almost constantly followed by extensive lesions in pulmonary arteries the alterations consisted of mural fibrinoid necrosis, sometimes with slight intramural occurrence of mononuclear inflammatory cells and eosinophils. Massive perivascular accumulations of the same cell types were also very common findings. Affected arteries were frequently occluded by precipitates, predominantly consisting of the injected material, which were rapidly replaced by proliferating endothelial cells, resulting in obliterative lesions, where giant cells sometimes occurred. The conclusion was drawn that the arterial lesions could most adequately be categorized as hypersensitivity angiitis.

Key words: Vascular lesions, pulmonary *Escherichia coli* cells; intravenous injections.

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In a preceding report, arterial renal lesions in various mammalian species, induced by systemic injections of disintegrated cells of *Escherichia coli* were described (13) The vascular lesions were found to be of the same type as those occurring in various organs in association with hypersensitivity to drugs and serum (4 8, 11 15 16, 21) hence, they were interpreted as a form of hypersensitivity angiitis, according to the description of *Zeek et al* (25–27) It was concluded that the pathogenetic mechanism was related to the Shwartzman phenomenon. Some animal species in the previous experiment did not develop renal arterial lesions. Among these latter animals, however some individuals in all species tested exhibited pulmonary mural arterial modifications, sometimes also arterial and/or venous thrombosis the pulmonary lesions were most pronounced in dogs (14) As mammals thus show some variation in the morphological response to systemic administration of crushed cells of *Escherichia coli* it was decided to perform a corresponding study in an avian species.

TABLE 1 *Treatment of Animals Used for the Illustrations*

Chicken No.	Doses, ml		Killed (K) or died (D) time after the second inject.
	First inject.	Second inject.	
3	1.0	1.0	K 18 hrs.
11	0.5	1.0	D 12 hrs.
16*	1.0	–	K 3 days
19‡	1.5	–	
21	0.8	0.8	K 24 hrs.
23	0.8	1.0	K 24 hrs.
29	1.0	1.0	K 2 days
33	0.8	0.6	K 8 days
41‡	1.5	–	

This animal received only one injection.
‡ These animals died in shock in few minutes after one injection.

MATERIAL AND METHODS

The strain of haemolytic *Escherichia coli* type 0 141 a b (NVH 2853) was originally isolated from pig intestine. The bacterial suspension was prepared by the same procedure as described previously (22) and frozen until used the suspension contained 0.0154 g dry matter per ml. A total of 48 female chickens (*Gallus domesticus*) of the White leghorn breed, about 4 weeks old and weighing 250–350 grams, were used as experimental animals. The animals received one or two injections, spaced 24 hours apart, given intravenously through the wing vein. Surviving animals were killed at different times after challenge (few min. – 8 days) by cervical fracture and bled by cutting the neck. Doses and times of killing, or death, of animals used as illustrative material are shown in Table 1. Two untreated chickens and animals injected with isotonic saline, were used controls.

All animals were autopsied and pieces of organs were fixed in a 10 per cent formaldehyde solution for at least 3 days, and/or in Zenker' and Carnoy's fixatives, embedded in paraffin and sectioned at about 5 μ. Sections were stained with haematoxylin and eosin (H&E) methyl green-pyronin, Wilder' silver tan, elastin an Gieson (eLvG) Di-PAS phosphotungstic acid haematoxylin (PTAH) the acid picro-Mallory method of Landrum and with the Martius scarlet blue (MSB) method (10). Formalin fixed frozen sections from the lungs were stained with Oil red O and Sudan III.

After centrifugation of the bacterial suspension the sediment was fixed in buffered 3 per cent glutaraldehyde and postfixed in 2 per cent osmic acid. Initial fixation for the electron microscopic study of the lungs of two animals was performed by injecting glutaraldehyde into the trachea and abdominal cavity. Lung pieces were thereafter fixed by the same procedure as the sediment and ultrathin sections from the latter material and the lungs were stained with lead citrate and examined in a Siemens Elmiskop I A.

RESULTS

Most chickens exhibited rapid respiration immediately after challenge. Some animals went into shock and died within a few minutes. Signs of the acute shock included severe dyspnoea with gasping, ruffling of feathers, stretching of the neck, muscular weakness and convulsions.

Macroscopic Lesions

In most animals, the lungs appeared congested and oedematous, whereas all other organs were unchanged.

Light and Electron Microscopic Investigations

1 *Control animals and injected material.* Lung sections from the controls revealed evident vacuolization of endothelial cells and distinct external and internal elastic membranes (Fig. 1). Electron microscopic examination of the bacterial material showed that the bulk consisted of easily recognizable bacterial remnants mixed with a homogeneous or slightly granular substance.

2 *Pulmonary arteries.* In animals dying

Fig. 1 A pulmonary artery from a control animal demonstrating vacuolization of endothelial cells and distinct internal and external elastic membranes e = erythrocytes. Staining: el.v G × 425

Fig 2 A dilated pulmonary artery occluded by partly laminated masses and aggregates of red cells. Chicken No. 19 H & E, × 100



shortly after the injections, several distended intrapulmonary arteries occluded by masses, which were frequently laminated, were found these masses consisted of a eosinophilic, homogenous material, mixed with minor irregular foci of erythrocytic aggregations (Fig 2) The precipitated homogenous material was strongly PAS positive, but was not positively stained by the fibrin staining methods. These lesions were found independently of whether the animals had been given one or two injections. The endothelium was flattened and greatly stretched, but obvious light microscopic endothelial injury was not ascertained at this stage of involvement. Electron microscopic examination showed that the precipitated occlusive material in the

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Fig 3 Ultrastructure of an arterial occlusion in immediate contact with the endothelium which is in an early stage of disintegration. Chicken No. 41 = endothelial nucleus. × 39000.



Fig. 4 Incipient proliferation of vacuolated endothelial cells in a pulmonary artery; the lumen is oc-
cluded by aggregated erythrocytes. Chicken No. 21 H & E, × 425

Fig. 5 Mural necrosis and occlusive thrombosis in a pulmonary artery; some erythrocytes can be iden-
tified within the fibrin-stained material. Chicken No. 3 PTAH, × 250.

Fig. 6 Mural necrosis in a pulmonary artery. The lumen is in large parts occluded by hyaline materia[l]
or an indefinite debris. The external elastic membrane is not obviously damaged, whereas the media
layer and elastica interna are destroyed in the lower part of the figure. Chicken No. 11; el v. G, × 4[2]5

Fig. 7 Mural fibrinoid necrosis in a pulmonary artery. The lumen is partly occluded by a hyaline ma-
terial which is stained as fibrin and has coalesced with the necrotic wall. Chicken No. 1. Acid phos-
Mallory method, × 680

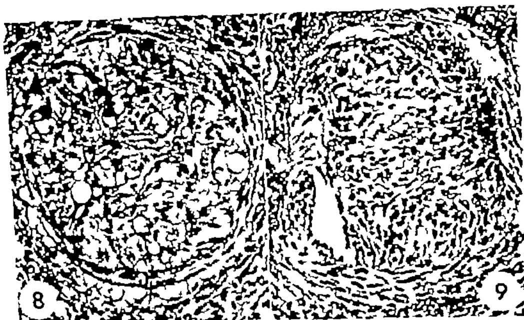


Fig 8 A pulmonary artery occluded by proliferating vacuolated cells, probably of endothelial origin, together with mononuclear inflammatory cells and collagenous tissue the vacuolated cells have penetrated the original vascular wall which is greatly reduced in thickness. Chicken No. 21 H & E, × 425.

Fig 9 A pulmonary artery which is almost occluded by proliferating fibro-cellular tissue. Chicken No. 16. Wilder' silver stain, × 340

arteries had an appearance which was very similar to that of the embedded bacterial sediment. There seemed, however at times to be some quantitative dissimilarities as the bacterial remnants, although always abundantly present, sometimes did not constitute as great a part of the deposits as in the injected material, while the homogenous or granular components were correspondingly increased. The precipitates were in intimate contact with the endothelium which was variably damaged (Fig 3)

The occlusive masses were gradually invaded by proliferating cells, probably of endothelial origin, containing PAS-positive granules. In somewhat later developmental stages, remnants of precipitated masses, or fibrin-stained thrombi, which frequently coalesced with the necrotic vascular walls, were found. Early mural lesions were, as a rule found in the endothelial layer and consisted of oedema, loss of endothelial nuclei and endothelial proliferative changes, with relatively large and highly vacuolated cells. Accumulations of lipid substances could not be demonstrated within these cells. Evident endothelial changes were also observed in arteries which were not occluded by precipitated bacterial material (Fig. 4)

The primary medial injury was localized in the subintimal areas and included degeneration and necrosis of smooth muscle cells when the lesions were extensive, the mural damage consisted of fibrinoid necrosis (Figs. 5–7) There was often however a marked difference between the stains used, as the PTAH method turned out to be negative in many areas where the hyaline material was positively stained with the acid picro-Mallory and the MSB methods. In advanced cases the internal elastic membrane was fragmented, discontinuous or totally destroyed. The lamina elastica externa was also affected in severe cases. Incipient intramural infiltration of mononuclear inflammatory cells and eosinophils was a common finding


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The necrotic areas of the medial walls were rapidly replaced by proliferating cells (Figs. 8-9); in some cases giant cells, of various morphological appearance were involved in the obliterative lesions (Figs. 10-11).

In the adventitia and the pulmonary tissue surrounding the affected arteries cellular accumulations frequently occurred. The great majority of the cells were immature mononuclear cells, with great variations in the nuclear sizes and chromatin content; some of the cells had considerable morphological similarities with plasma cells and were pyroninophilic. Eosinophils were frequently present, and the cellular infiltrations consisted occasionally predominantly of eosinophilic cells. In several cases apparently unaffected or moderately damaged arteries were also circumscribed by cellular accumulations (Figs. 12-14) or oedema. The remaining pulmonary tissue was congested; any precipitates of material resembling that occurring in the arteries were not found in the blood capillaries.

The arterial lesions and perivascular cellular accumulations appeared to be qualitatively independent of whether the animals had been given one or two injections of the bacterial material, and seemed to increase with the length of time after the inoculations. Animals dying in shock after a second injection frequently showed both acute occlusive lesions and perivascular alterations. Only pulmonary vessels were affected, whereas the bronchial arteries were spared.

3. Extrapulmonary tissue. Evident vascular lesions comparable to the changes in the lungs were not noted in extrapulmonary sites, including the kidneys.

DISCUSSION

Avian lungs are anatomically quite different from those of mammals, but their vascular system is similar (1). The present investigation demonstrates that disintegrated cells of *Escherichia coli* induce necrotic mural arterial lesions in the lungs of chickens and arterial obliterative modifications, following occlusions by precipitates of the injected material, when this is administered intravenously. As endothelial proliferation and perivascular accumulation of inflammatory cells were commonly present in the absence of occlusive lesions, one may probably conclude that this precipitation was not a prerequisite for the mural damage. The arterial alterations may therefore be interpreted as equivalent to the arterial injury observed in the kidneys (13) and in the lungs (14) in previous experiments in which mammalian experimental animals were injected with the same bacterial material. As some of the animals in the former experiments developed renal cortical necrosis, and as pulmonary vascular involvement is quite common in association with hypersensitivity reactions in man (6, 7, 24), the findings in this experiment tend to support the view that hypersensitivity plays a role in the development of the generalized Shwartzman reaction in mammals.

The injected material appeared to constitute the main component of the arterial precipitates, but it seems likely that plasma proteins were also included to a certain extent. As considerably higher doses of this material, if the body weights are taken into account, have been injected intravenously into mammals (mice) without corresponding arterial occlusions, one may conclude that the

Figs. 10-11. Giant cells of various morphological types in obliterative lesions in pulmonary arteries. Fig. 10. Chicken No. 33. e = erythrocytes. H & E, × 425. Fig. 11. Chicken No. 23. H & E, × 425.

Fig. 12. Massive proliferation of mononuclear cells around a pulmonary artery which is almost occluded (arrow). Chicken No. 16. H & E, × 190.

Fig. 13. Cellular accumulation in the neighbourhood of two pulmonary arteries. t = tertiary bronchus. Chicken No. 16. H & E, × 170.

Fig. 14. A pulmonary artery with an irregular cellular pattern, circumscribed by predominantly mononuclear cells. = erythrocytes. Some eosinophils are indicated by arrows. Chicken No. 29. H & E, × 680.


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LOSS OF EPITHELIAL BLOOD GROUP ANTIGENS A AND B IN ORAL PREMALIGNANT LESIONS

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Dabelsteen, E., Roed Petersen B. & Pindborg, J J Loss of epithelial blood group antigens A and B in oral premalignant lesions. Acta path. microbiol. scand. Sect. A, 83 292 300, 1975.

Tissue from 40 oral premalignant lesions were investigated for the presence of blood group antigens A and B. The material included 18 leukoplakias, 1 erythroplakia, and 3 lichen planus, all with varying degrees of epithelial dysplasia, and 18 leukoplakias without histological evidence of impending malignancy Thirty-eight benign keratotic oral mucosal lesions were included as a control group. The antigens were demonstrated by a double layer immunofluorescence technique, and the reactivity was compared by titration, to the reactivity of adjacent normal epithelium from the same patient. All 22 lesions with dysplasia showed decreased reactivity for blood group antigen. Among the 18 leukoplakias without any signs of impending malignancy 4 cases demonstrated loss of antigen reactivity None of 38 benign control lesions showed any change in antigen reactivity as compared to normal adjacent epithelium

Key words Blood group antigen A and B epithelium oral premalignant lesions.

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The term *epithelial dysplasia* of the oral mucosa is generally used to describe histological changes in the epithelium which are believed to have malignant potential (*Kramer* 1973 *Pindborg et al.* 1963) Epithelial dysplasia is most often found in oral leukoplakias and oral erythroplakias (*Pindborg et al.* 1968, *Shear* 1972) Recent studies, however indicate that only some lesions diagnosed as epithelial dysplasia actually develop into cancer and that cancer may in some cases develop in a leukoplakia without any recognizable interphase of dysplasia (*Brocheriou et al* 1973 *Mincer et al* 1972 *Pindborg & Roed Petersen* 1973) Besides the histological changes certain *clinical features are known* to be precancerous but even when such characteristics are present we have no way of telling whether cancer will develop and when

Many human tumours are known to have tumour-associated antigens (*Hellström & Hellström* 1971) and furthermore the acquisition of tumour antigen is sometimes accompanied by loss of normal cytoplasmic antigens (*Burtin et al* 1972 a)

Lappé (1969) and *Halpin et al.* (1972) have demonstrated that the antigen changes present in murine premalignant lesions can persist unchanged through progression to malignancy Likewise, preliminary studies on the blood group antigens, which are normally present on the cell membranes of oral epithelial cells, have shown that these disappear

TABLE 1 *Data of Patients Having Oral Lesions with Epithelial Dysplasia*

Patient/ Lesion	Sex	Age	Blood group	Clinical diagnosis	Localization	Degree of dysplasia	Endpoint titre	
							Normal	Lesion
1	♀	71	B	Eryth.	Buccal	Slight	1:16	0/1:16
2/a	♂	54	A	Leuk.	Lower lip	Moderate	1:256	0
2/b		54		Leuk.	Tongue	Moderate	1:256	0
3	♂	69	A	Leuk.	Lower lip	Slight	1:256	0
4	♂	49	A	Leuk.	Commissure	Slight	1:32	0
5	♀	60	A	Leuk.	Lower lip	Severe	1:1	0/1:1
6	♀	59	AB	Leuk.	Buccal mucosa	Slight	1:4	0/1:4
7	♂	73	A	Leuk.	Commissure	Slight	1:16	0
8	♂	71	A	Leuk.	Commissure	Slight	1:4	1:4
9	♂	55	A	Leuk.	Buccal mucosa	Slight	1:32	0
10	♀	67	A	Leuk.	Buccal mucosa	Moderate	1:32	0
11	♀	73	AB	Leuk. Carc.?	Buccal mucosa	Moderate	1:16	0
12	♂	66	A	Leuk. Carc.?	Floor of the mouth	Carc.-in-situ	1:128	0/1:16
13	♂	66	A	Leuk.	Floor of the mouth	Slight	1:128	0
15	♀	40	A	Leuk.	Floor of the mouth	Slight	1:128	0
17	♀	76	A	Leuk.	Tongue	Slight	1:128	0
18/a	♀	60	A	Lichen	Tongue	Slight Carc.?	1:256	0
18/b	–	60	–	–	Buccal mucosa	Slight Carc.?	1:256	0
19	♀	46	B	Lichen Leuk. Carc.?	Floor of the mouth	Severe	1:32	0
20	♂	53	A	Leuk.	Floor of the mouth	Severe	1:64	0
21	♂	62	B	Carc.?	Buccal mucosa	Carc.-in-situ + (Carc.)	1:128	0
22	♂	75	A	Leuk.	Buccal mucosa	Moderate	1:32	0

Eryth. Erythroplakia, Leuk. Leukoplakia, Carc. Carcinoma.
endpoint titre in patchy areas.

in most oral carcinomas (*Dabelsteen & Pindborg* 1973) and in some oral lesions with epithelial dysplasia (*Dabelsteen & Fulling* 1971). The present investigation was designed to determine further changes in blood group antigen expression in oral premalignant lesions. The hope is that changes in expression of these antigens may prove useful prognostic features in follow-up studies.

MATERIAL AND METHODS

Leukoplakias were in the present study defined according to the criteria given by *Pindborg et al.* (1963) and erythroplakia according to the criteria given by *Shear* (1972).

The material was divided into three groups.

1) *Lesions with Epithelial Dysplasia*

Nineteen patients with 22 lesions were investigated. The distribution of sex, age, blood group, clinical diagnosis and localization is given in Table 1. All patients had a biopsy taken from the lesion. Eleven of the patients had a biopsy taken from clinically normal mucosa—lower lip or contralateral to the lesion—as well. In the remainder of the cases histologically normal mucosa was included in the same biopsy as the dysplastic epithelium. The normal mucosa was obtained to allow comparisons between blood group antigen reactivity in dysplastic and normal mucosa of the same patient (*Dabelsteen* 1972).

In nine of the patients follow-up biopsies of the dysplastic lesions (test lesions) were investigated as well. The follow-up period was between 6 months and 10 years (Fig. 5).

TABLE 2. *Data of Patients Having Oral Premalignant Lesions without Any Histological Evidence of Potential Malignant Development*

Patient No.	Sex	Age	Blood group	Localisation of leukoplakia	Endpoint titre	
					Normal	Leukoplakia
23	♂	44	A_2	Commissure	1:32	1:32
24	♀	61	A_1B	Buccal mucosa	1:64	1:64
25	♀	63	A_2	Buccal mucosa	1:64	1:64
26	♂	64	A_1	Buccal mucosa	1:32	1:32
27	♀	54	A_1	Buccal mucosa	1:128	1:128
28	♀	59	A_1	Buccal mucosa	1:128	1:1
29	♀	48	A_1B	Buccal mucosa	1:64	1:64
30	♀	73	A_1B	Buccal mucosa	1:16	0
31	♂	73	A_1	Buccal mucosa	1:32	1:32
32	♀	48	A_1	Commissure	1:512	1:256
33	♂	47	A_1	Buccal mucosa	1:64	1:32
34	♂	82	A_1	Lower lip	1:128	1:256
35	♂	73	A_1	Buccal mucosa	1:32	1:16
36	♀	47	A_1	Buccal mucosa	1:2	1:4
37	♂	67	A_1	Buccal mucosa	1:32	1:32
39	♂	55	A_1	Buccal mucosa	1:32	0
40	♀	74	A_1	Buccal mucosa	1:256	1:32
41	♂	69	A_1	Lower lip	1:32	1:32

TABLE 3. *Blood Typing of Tissue from Benign Oral Keratotic Lesions. Immunofluorescence Staining*

Blood group antisera	Anti-A test serum	Anti-A test serum absorbed	Anti-B test serum	Anti B test serum absorbed	Phosphate buffered saline
Conjugate	Antihuman IgG/FITC	Antihuman IgG/FITC	Antihuman IgG/FITC	Antihuman IgG/FITC	Antihuman IgG/FITC
Results of cases typed as A	+	—	—	—	—
Result of a case with known A reactivity	+	—	—	—	—
Result of a case with known B reactivity	—	—	+	—	—

2) *Leukoplakias without Any Histological Evidence of Potential Malignant Development*

Nineteen patients (Nos. 23–41) were investigated. The distribution of sex, age, blood group and localisation of leukoplakia is given in Table 2. All patients had a biopsy taken from the leukoplakia and from clinical normal mucosa of the lower lip. The normal mucosa was included for the same reason as mentioned above.

3) *Benign Keratotic Lesions of the Oral Mucosa*

This group included 38 fibroepithelial polyps covered with keratinized as well as unkeratinized epithelium. The keratinization of these lesions was believed to have resulted from mechanical trauma. The patients were between 6–78 years with 16 over 42 years. All belonged to blood group A.

Blood Grouping of Patients

Blood grouping was performed at Statens Serum Institut, Copenhagen, for the patients in groups 1 2.

As no blood groups were available for the 38 control lesions, blood grouping was done by immunofluorescence staining of sections of the formalin-fixed, paraffin-embedded surgical material. The readings were made on the unkeratinized buccal epithelium which was included in all of the surgi-



Fig 1 Borderline between normal and dysplastic epithelium. A. Haematoxyline and eosin staining. Normal epithelium to the right. Dysplastic epithelium to the left. B. Immunoperoxidase staining of neighbouring section. Black intercellular spaces indicate the presence of blood group antigen these are only present in the normal epithelium. × 60

cal specimens. The sera used in this test are described below and the blood grouping was performed according to Table

Handling of Tissue

Local infiltration anaesthesia (2 per cent lidocaine noradrenaline) was used and care was taken not to inject directly into the site of biopsy

The biopsies were fixed in 10 per cent neutral formalin, paraffin embedded, and sectioned at 5 μm. The histology of the tissue was investigated on H & E-stained sections. The criteria for epithelial dysplasia have been described previously (*Dabelsteen et al.* 1971)

The degree of epithelial dysplasia was graded as slight, moderate, severe or carcinoma-in-situ. All the sections were assessed independently by two of the authors (*Dabelsteen & Pindborg*) The defects of such a subjective procedure are recognised, but in the absence of any accurate method for the quantitation of epithelial dysplasia, it was decided to follow the conventional subjective assessment used in routine histological diagnosis.

Detection of Blood Group Antigen

Tissue from group A and AB persons were examined for blood group antigen A and the tissue from group B persons for blood group B.

Blood group antigens in the epithelium were in all cases detected by the immunofluorescence (IF) staining. When adequate tissue was available immunoperoxidase (IP) staining was performed as well (*Weller & Coons* 1954 *Avrameas* 1969 *Dabelsteen & Rygaard* 1972) Both methods were used as double-layer techniques—blood group antiserum, conjugate control reactions and fluorescence microscopy were employed as described previously (*Dabelsteen* 1972) The amount of antigen in the tissue was estimated by a two-fold titration of the IF staining dilutions from 1 1 up to 1 512 were used. *The IP stainings were performed only with* blood group antiserum in 1 2 dilutions. Tissues to be compared were always stained simultaneously

RESULTS

In all cases the normal mucosa reacted positively with bright green fluorescence at the cell membrane in the spinous cell layer

Group 1

In Case No. 5 the normal mucosa reacted very weakly As any decrease in reactivity in this case would thus be difficult to measure because of the uncertainty in the staining reaction, it was excluded from the study

A total loss of blood group antigen in the dysplastic epithelium was seen in 16 cases. In 3 cases the majority of cells reacted negatively but some positive areas were always found (Fig 1)

In only one case (No. 8) did the dysplastic epithelium demonstrate no change in blood group antigen A reactivity No correlation was found between the degree of severity of the dysplasia and the blood group antigen changes (Table 1)

The results from the ten cases with follow-up are summarized in Figs. 2 and 3 It is seen

A HISTOLOGY

latest biopsy \ first biopsy	N	Sl	M	Se	C
N					
Sl		15,7			
M	18 b	8 3	11		
Se				1	
C		13 17 18 a			

N no dysplasia Sl slight dysplasia; M moderate dysplasia Se severe dysplasia C cancer

B BLOOD GROUP ANTIGEN

latest biopsy \ first biopsy	N	D	L
N			
D			
L	8 18b	1 17	3 7 11 13 15 18a

N as normal mucosa D decreased L lost

Fig 2 Results of follow-up investigation. Each number represents a case number and the same number is used for the same case in all the figures and tables. A. Histology of first and latest biopsy B. Antigen reactivity in first and latest biopsy

CASE NO	1	3	7	8	11	13	16	17	18a	18b
YEARS	2	1	½	3	2	6	1	10	1	9

Fig 3 Period between first and latest biopsy The same number is used for the same case in all the figures and tables.

that Case No. 8 which in the first biopsy demonstrated slight dysplasia and no blood group antigen change in the follow-up biopsy showed a loss of blood group antigen and increased at the same time from slight to rate dysplasia.

Three cases developed into carcinoma during the follow up period. Case No. 15 developed from a lesion with slight dysplasia to carcinoma with adjacent carcinoma-in-situ. In the first as well as in the follow-up biopsy a total loss of blood group antigen was noted Two cases (Nos. 17 and 18a) were diagnosed as hyperplastic epithelium with slight dysplasia. One of these cases occurred at the site of a previously excised squamous carcinoma. Both cases demonstrated decrease in blood group reactivity Follow up biopsies (Case No. 17 10 years later Case No 18a 1 year later) revealed squamous cell carcinoma in both instances with total loss of blood group reactivity Case No. 18 b developed from no to moderate dysplasia over a 9-year period and changed at the same time from a "normal" antigen reactivity to a total loss. The other cases demonstrated no change in histology or blood group reactivity

Group 2 and 3

A total loss of blood group A was seen in three leukoplakias and in one further case the antigen expression was so weak that it could hardly be detected. The difference in *endpoint* titre between the leukoplakia and the normal epithelium ranged in these cases between five and eight two-fold titre steps. In one case the leukoplakia reacted positively but with an endpoint titre three two-fold titre steps lower than the normal mucosa from the same patient. In the remainder of the samples only a slight variation between

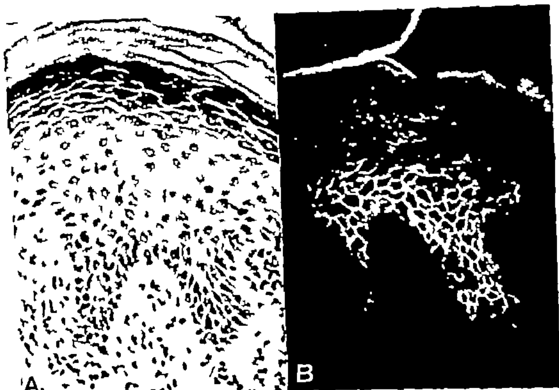


Fig 4 Leukoplakia without any histological evidence of malignancy A. Haematoxyline and eosin. B. Immunofluorescence of neighbouring section. The bright intercellular spaces in the spinous cell layer indicates the presence of blood group antigen. × 90.

leukoplakic and normal mucosa could be found (Table 2)

In none of the controls (lesions from group 3) was there a variation of blood group antigen reactivity in keratinized and unkeratinized epithelium greater than one endpoint titre (Fig 4)

A previous estimation of the reproducibility of the endpoint titre by triple titration of the same specimen in 21 cases (63 stainings) has not given any variation between first, second, and third staining which was greater than two two-fold titration steps. By using this limit and a Fischer's exact test (*Hald* 1956) a significant difference ($p < 0.01$) was found between the leukoplakic material and the fibroepithelal polyps used as control tissue.

DISCUSSION

The present work has investigated the expression of blood group antigens A or B in oral premalignant lesions compared with the antigen expression in uninvolved oral mucosa of the same patients.

Previous studies (*Dabelsteen* 1972, *Dabelsteen & Fejerskov* 1974 a) have shown the existence of interindividual as well as regional variation in reactivity of blood group antigen in normal epithelium of the oral cavity In group 2 comparisons were made only between blood group antigen reactivity of labial, buccal, and commissural epithelium. The reactivity of normal epithelium from these three regions have been shown to be of the same magnitude (*Dabelsteen & Fejerskov* 1974 a) In group 1 antigen reactivity of the epithelium of the floor of the mouth was compared with that of the lip in two cases.

It appeared justified because unpublished data indicate that the blood group antigen reactivity of the epithelium from the buccal mucosa and the floor of the mouth are of the same magnitude.

In ten of the cases in group 2 the antigen reactivity in the dysplastic epithelium was compared with the reactivity of adjacent normal epithelium. Previous studies (*Dabelsteen & Pindborg* 1973 *Dabelsteen et al* 1974) have demonstrated that histologically normal epithelium adjacent to carcinomas may in a few cases, demonstrate blood group antigen changes. The mucosa used as normal control should thus be obtained distant from the lesion. However in the ten cases in group 2 this was not possible for different practical reasons.

As all the oral leukoplakias are keratinizing processes, a benign keratotic lesion of the buccal mucosa was included in the study as a control group to the leukoplakias. By doing so it was possible to investigate whether antigen changes in leukoplakias were simply the result of the keratinizing process.

Premalignant lesions are usually regarded as constituting a spectrum with insignificant epithelial disturbances at one end and late precancerous lesions or early carcinoma at the other end. Borderlines between the different categories are sometimes difficult to determine. When the sample investigated is divided into lesions with antigen reactivity as in normal epithelium and lesions with loss or decrease in antigen reactivity it is seen that the cases with decreased or lost reactivity include the dysplastic lesions plus four lesions without any histological signs of malignancy. It will be of great interest to see whether these five cases are more likely to develop into dysplasia and cancer than the rest of the leukoplakias without severe epithelial changes.

It is of interest to notice that two of the four lesions are from patients who have epithelial dysplasia and loss of blood group antigen in other lesions of the oral cavity.

The present findings are especially of interest when one considers the work of *Prehn & Slemmer* (1963) *Lappe* (1969) and *Halpin et al.* (1972) who have demonstrated in animal experiments that the antigenic properties of preneoplastic cell populations are carried over into the resulting tumours after the neoplastic transformation has occurred and that antigenicity or lack of it is a property that is already established in the preneoplastic stages of tumour development.

That only some of the leukoplakias showed antigen changes corresponds to previous studies which have demonstrated cell-mediated hypersensitivity to some, but not all, leukoplakias (*Roed Petersen et al.* 1973). The lesions with antigen changes in the present study have not been investigated for demonstration of cellular hypersensitivity.

Although the relationship between epithelial dysplasia in a leukoplakia and malignant transformation of the lesion is debatable, many workers consider that the finding of epithelial dysplasia indicates a likelihood that malignancy will develop. On this background it should be noted that all the dysplastic lesions in the present study demonstrate loss of blood group antigen. It may be possible that all the dysplastic lesions investigated have true malignant potential but that the prognosis of the lesions is dependent on extraepithelial factors such as the host immunological responsiveness. Host reactivity against oral leukoplakias with epithelial dysplasia has actually been demonstrated by *Roed Petersen et al.* (1973) and by *Lehner* (1970) although in few patients only.

It is, however more probable that the antigen changes found in the dysplastic lesions are associated with other factors, such as cell movement and growth rate, rather than malignancy per se. That blood group antigen expression and neoplastic transformation are not necessarily interdependent is demonstrated by the presence of blood group antigens in a few oral carcinomas and by the loss of antigens on epithelial cells taking part in wound healing (*Dabelsteen & Fejerskov* 1974 b).

In the six lesions where follow-up biopsies demonstrated a change to a more severe di-

ease it was interesting to notice that the antigen changes were already present at the lowest stage of morphological change. However a larger sample, improved criteria for the diagnosis of epithelial dysplasia, and very long follow up studies are needed before a definite conclusion can be made.

The present findings are in agreement with *Burtin et al.'s* (1972 b) studies on premalignant lesions of the colon. He investigated undifferentiated non-invasive polyps for three different antigens and concluded that from an immunological viewpoint polyps behave as cancer. Furthermore, *Burtin et al.* (1972 a) found that the loss of normal cytoplasmic antigens in colon tumours were accompanied by the appearance of new tumour associated antigen. We have not investigated whether the loss of blood group antigens in the dysplastic lesions is accompanied by the appearance of new tumour associated antigens, against which immunity may exist, but such an investigation would of course be of interest.

In conclusion the present study has demonstrated that it is not possible by investigating the distribution of blood group antigens in oral lesions with dysplasia to divide these into different groups. However by dividing oral precancerous lesions into lesions with antigen reactivity as in normal epithelium and lesions with loss or decrease in antigen reactivity it is seen that the cases with decreased or lost reactivity include the dysplastic lesions plus some lesions without any histological signs of malignancy. These findings may be of prognostic value.

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It appeared justified because unpublished data indicate that the blood group antigen reactivity of the epithelium from the buccal mucosa and the floor of the mouth are of the same magnitude.

In ten of the cases in group 2 the antigen reactivity in the dysplastic epithelium was compared with the reactivity of adjacent normal epithelium. Previous studies (*Dabelsteen & Pindborg* 1973 *Dabelsteen et al.* 1974) have demonstrated that histologically normal epithelium adjacent to carcinomas may in a few cases, demonstrate blood group antigen changes. The mucosa used as normal control should thus be obtained distant from the lesion. However in the ten cases in group 2 this was not possible for different practical reasons.

As all the oral leukoplakias are keratinizing processes, a benign keratotic lesion of the buccal mucosa was included in the study as a control group to the leukoplakias. By doing so it was possible to investigate whether antigen changes in leukoplakias were simply the result of the keratinizing process.

Premalignant lesions are usually regarded as constituting a spectrum with insignificant epithelial disturbances at one end and late precancerous lesions or early carcinoma at the other end. Borderlines between the different categories are sometimes difficult to determine. When the sample investigated is divided into lesions with antigen reactivity as in normal epithelium and lesions with loss or decrease in antigen reactivity it is seen that the cases with decreased or lost reactivity include the dysplastic lesions plus four lesions without any histological signs of malignancy. It will be of great interest to see whether these five cases are more likely to develop into dysplasia and cancer than the rest of the leukoplakias without severe epithelial changes.

It is of interest to notice that two of the four lesions are from patients who have epithelial dysplasia and loss of blood group antigen in other lesions of the oral cavity.

The present findings are especially of interest when one considers the work of *Prehn & Slemmer* (1963) *Lappe* (1969) and *Ha-pin et al.* (1972) who have demonstrated i animal experiments that the antigenic prop- erties of preneoplastic cell populations a carried over into the resulting tumours afte the neoplastic transformation has occurre and that antigenicity or lack of it is a prop- erty that is already established in the preneo- plastic stages of tumour development.

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MORPHOMETRIC STUDIES OF THE JUXTAGLOMERULAR APPARATUS IN PERIMEMBRANOUS GLOMERULONEPHRITIS

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I 23 cases of perimembranous glomerulonephritis (PGN membranous nephropathy) the juxtaglomerular cell complex (JGC-complex) in early postmortal (2 cases) and bioptically obtained specimens (21 cases) was investigated quantitatively by planimetry The results were correlated with the stage of the PGN and the clinical findings. A statistically significant increase in mean size of the JGC-complex was found in perimembranous glomerulonephritis compared with the control group ($p < 0.005$) Comparing the different morphological stages of PGN the size of the JGC-complex was increased and the serum creatinine level raised in stages 3 and 4 In hypertensive patients with perimembranous glomerulonephritis, the serum creatinine levels were significantly raised as compared with the normotensive group

Key words Juxtaglomerular apparatus perimembranous glomerulonephritis.

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The juxtaglomerular apparatus (JGA) consisting of various structures at the vascular pole of the glomerulus (Fig 1) is considered to be the site of renin production. Renin is found to be mainly located in the epithelioid, granulated cells in the wall of the afferent arteriole (*Bing et al.* 1967 *Faarup* 1968). Four principal mechanisms are generally regarded to be involved in the control of renin secretion The pressure or tension exerted on the wall of the afferent arterioles (baroreceptor hypothesis, *Tobian* 1960) the sodium concentration or the sodium load in the distal tubule (Macula densa theories, *Thurau* 1965 *Lander* 1967) the sympathetic nervous system and the circulating levels of catecholamines (*Lander* 1965) Furthermore, angiotensin exerts a negative feedback effect on production and release of renin from the kidney (*Lander et al* 1965) It must be mentioned, however that several investigators have also postulated that other factors are involved in renin secretion.

The changes in tension of the afferent arteriolar wall are not only affected by the arterial pressure *per se* but also depend on variations of the intravascular fluid volume. Diseases involving a reduction of the "effective" blood volume, as in nephrotic syndrome, would therefore be expected to stimulate renin production. In perimembranous glomerulonephritis, proteinuria is always present and in 60 per cent of the cases, nephrotic syndrome and hypertension are also found (*Reichel et al.* 1973) Using diagnostic light microscopy the impression is obtained that the JGA is larger in PGN than in normal

kidneys. Therefore, we felt the need for a morphometric study of the juxtaglomerular apparatus in this disease.

MATERIAL AND METHODS

Our investigations were carried out on renal tissue obtained by wedge biopsy or at early postmortal autopsy because the usual interval between death and fixation gives inaccurate results due to autolytic changes. Material from percutaneous needle biopsy specimen is unsuitable for our method of investigation because of the insufficient number of extracted glomeruli and adjacent JGA. This explains the limited number of specimens in this study as we could not use all our biopsy material including more than 300 cases of PGN but only the small number of 23 wedge biopsies and samples obtained at early postmortal autopsies. All tissue samples were fixed in 4 per cent formaldehyde solution, and the deparaffinized sections, 5–6 μ in thickness, were stained with periodic acid-Schiff stain. The planimetric examination was carried out first, and only afterwards were the results correlated with the clinical data. The light microscopical diagnosis of perimembranous glomerulonephritis was confirmed in all cases by means of 1 μ-sections from plexiglass-embedded renal tissue stained with periodic acid silver methenamine (PASM) according to MOVAT

According to the classification given by *Ehrenreich & Churg* (1968) the cases were grouped into stages 1–4

Stage 1

The extra- or perimembranous deposits on the glomerular capillaries are small. Some capillaries can be entirely free of deposits. The spikes, located between the deposits, are also few The capillary basement membrane is normal.

Stage 2

The deposits are much more numerous and in some areas "contiguous" Many of the spikes between the deposits show lateral extensions. Only focally the basement membrane is irregular

Stage 3

At this stage, the spikes extend laterally thus leading to an incipient incorporation of the numerous deposits into the basement membrane. There is now an irregularity and focal thickening of the basement membrane.

Stage 4

Almost all deposits are incorporated and the basement membrane is inhomogeneous and thickened. But the thickening is not uniform.

In their search for a morphological parameter of renin production in the JGA, *Hartroft & Hartroft* (1953) used, in rat experiments, the abundance of secretory granules in the juxtaglomerular cells as a semiquantitative indicator to assess renin production. Their Juxtaglomerular Granulation Index (JGI) can hardly be applied to human renal tissue because the JG-cell granules themselves are very small and difficult to identify by light microscopy and because lipofuscin granules can easily be mistaken for so called specific "renin granules" (*Biava & West* 1965 *Bohle & Sitte* 1966)

Turgeon & Sommers (1961) introduced the "Juxtaglomerular Cell Count" (JGCC) using the cellularity of the JGA to describe the functional state of the renal renin system.

In this study we measured the mean cut area of the juxtaglomerular cell complex (JGC-complex), i.e. the epithelioid cells of the vas afferens, and occasionally the vas efferens, and the Goormaghtigh cell group (*Meyer* 1972) We have the impression that, in diseases involving an increased renin production, the enlargement of the JGA is based on an increased number of epithelioid cells in the wall of the afferent arteriole and hyperplasia and apparent transformation of the Goormaghtigh cell group into epithelioid-like cells. An increased number of all cells of the JGA, except the cells in the macula densa, has been found in pathological states such as renal artery stenosis, Addison's disease and pseudo-Bartter-syndrome (*Halber et al* 1970)

For our quantitative studies of the JGC-complex we use the planimetric method as described by *Meyer* (1972) a simple and sufficiently exact method for comparative investigations of the JGC complex in various pathological states of the renin system. We determine the mean cut area of 50 randomly selected JGC-complexes in *PAS-stained* sections. Projecting the sections with a ZEISS-Projections-Zeichengerat at a 560 *fold enlargement*, the projected and *delineated areas are measured* planimetrically with an OTT Planimeter The Wilcoxon's rank correlation was used for the statistical analysis.

RESULTS

The main clinical features and the results of the measurements of the JGC-complexes in 23 patients with perimembranous glomerulonephritis are summarized in Table 1 The average age of the patients was 40 years varying from 14 to 67 years. Two-thirds of the patients were males. Proteinaemia varied from 3.9 to 6.3 g/100 ml with a mean value of 5.2 g/100 ml. The mean serum creatinine level of 2.9 mg per cent was markedly raised

TABLE 1 *Clinical and Laboratory Data and the Size of the JGC-Complex in 23 Patients with Perimembranous Glomerulonephritis*

Case no.	Sex	Age at invest. (years)	Hist. stage	Mean size of JGC-complex (μ^2)	Blood pressure (mm Hg)	Serum creatinine (mg/100 ml)	Proteinaemia (g/100 ml)
1	F	53	1	942.0	130/85	0.88	4.5
2	F	20	1	1010.6	110/70	0.7	6.0
3	F	44	1	1031.0	120/70	N	5.4
4	F	14	1	1040.0	120/60	3.4	6.2
5	M	51	1	937.2	110/80	0.85	L
6	M	30	1	1104.0	120/80	–	4.8
7	M	60	1	1250.0	140/90	1.4	5.3
8	F	51	2	928.2	155/110	0.9	4.7
9	F	34	2	949.4	–	–	–
10	M	18	2	831.8	130/60	1.35	5.6
11	M	48	2	904.0	125/80	2.0	4.8
12	M	41	2	1125.2	–	R	L
13	M	38	3	948.6	180/100	6.8	4.2
14	M	47	3	1007.4	140/80	1.8	5.0
15	M	46	3	1076.6	160/110	2.3	5.2
16	M	34	3	1052.0	130/100	1.21	5.7
17	M	28	3	1095.0	R	R	–
18	M	67	3	1182.6	150/90	1.1	6.3
19	M	40	3	1296.0	180/100	2.9	5.9
20	M	40	3	1495.4	140/90	1.4	L
21	F	20	4	1114.6	110/80	N	5.8
22	M	32	4	1118.4	160/110	15.0	5.5
23	M	59	4	1251.4	180/120	5.8	4.8

M = Male; F = Female N = Normal R = Raised L = Lowered.

TABLE 2 *The Mean Clinical Values and the Size of the JGC-Complex in Normotensive and Hypertensive Cases of Perimembranous Glomerulonephritis*

	Perimembranous glomerulonephritis	
	Normotensive group (n = 15)	Hypertensive group (n = 7)
Mean age at time of investigation	39 years	43 years
Mean duration of the disease	2 years, 1 month	3 years
Mean size of the JGC-complex cut area	1067 ± 171 μ^2	1089 ± 142 μ^2
	(S. $p > 0.2$)	
Mean blood pressure	130/80 mm Hg	165/110 mm Hg
Mean arterial blood pressure	102 ± 10 mm Hg	135 ± 10 mm Hg
	(S. 2 $p < 0.001$)	
Mean proteinaemia	5.4 g/100 ml	4.9 g/100 ml
Mean serum creatinine level	1.3 mg/100 ml	5.0 mg/100 ml
	(S. 2 $p < 0.001$)	

S = Significance.

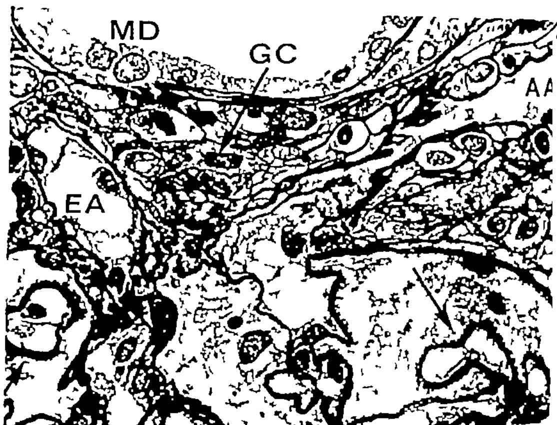


Fig 1 Case 8. The relationship of the different structures of the JGA at the vascular pole of the glomerulus. The cell group of Goormaghtigh (GC) is shown in the triangle formed by the afferent arteriole (AA) the efferent arteriole (EA) and the macula densa (MD) In this case the cell group of Goormaghtigh consists of apparently hypertrophied cells of transitional form, some of them with an epithelioid cell-like appearance. In the wall of the afferent arteriole (AA) large, round and pale epithelioid cells can be seen. On the glomerular capillaries the spikes between the deposits are almost contiguous in some areas (arrows) Perimembranous glomerulonephritis stage 2. PASM, × 1250.

As shown in Fig 2, the JGC-complex of PGN exhibits a statistically significant increase in mean size. The size in the individual cases varies between 832 μ and 1496 μ^2 with a mean value of 1072 ± 148 μ^2 ($\bar{x}$ ± S.D) for the whole group As the number of patients in each morphological stage is too small, the average size of the JGC-complex in PGN was determined for all cases together regardless of stage. The control group consisting of histological specimens from 10 normotensive patients with normal kidney tissue, showed a JGC-complex mean cut area of 919 ± 54 μ In our study the JGC-complex in the stages 3 and 4 of PGN proved to be *significantly larger than in the stages 1* and 2 ($p < 0.025$)

As demonstrated in Table 2, the patients are classified into a normotensive and a hypertensive group without taking into account the therapy applied It is of special interest that the JGC-complex in the normotensive (1067 ± 171 μ) and hypertensive group (1089 ± 142 μ) is of almost equal size ($2p > 0.7$) The significant increase in the mean serum creatinine level in the hypertensive patients to 5 0 mg per cent in comparison with 1.5 mg per cent in the normotensive should be noted ($2p < 0.001$)

Fig 3 relates the mean blood pressure to the morphological stage of the PGN. The mean arterial blood pressure in stages 3 and 4 (*put together as one group*) is found to be significantly increased as compared with

Fig. 2 A graphic representation of the relation between the size ($\bar{x}$, S.D.) of the JGC-complex (μ^2) and the morphological stages of perimembranous glomerulonephritis in comparison with the average size of JGC-complex in normal kidneys ($\bar{x} \pm$ S.D.)

stages 1 and 2 ($p < 0.005$). Evaluated in the same way significantly higher levels of serum creatinine were found in stages 3 and 4 ($p < 0.025$).

DISCUSSION

The importance of the different factors involved in the control of the renin-angiotensin-aldosterone-system (RAAS) is not clear though a large number of investigations concerning the RAAS under different pathological conditions have been published during the last few years. The main problem seems to be the number of parameters involved in the control mechanisms. Hypovolaemia is generally accepted to be an important stimulus for renin secretion. In diseases involving generalized oedema such as the nephrotic syndrome there are changes of blood volume and investigators have found a partly marked

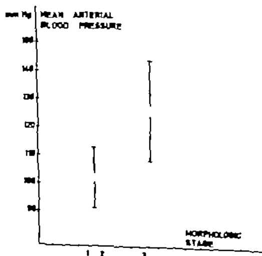


Fig. 3 A graphic comparison between the mean arterial blood pressure and the morphological stages of the perimembranous glomerulonephritis (Stage 1 + 2 $\bar{x} \pm$ S.D. = 102 ± 11 mm Hg, Stage 3 + 4 $\bar{x} \pm$ S.D. = 126 ± 18 mm Hg)

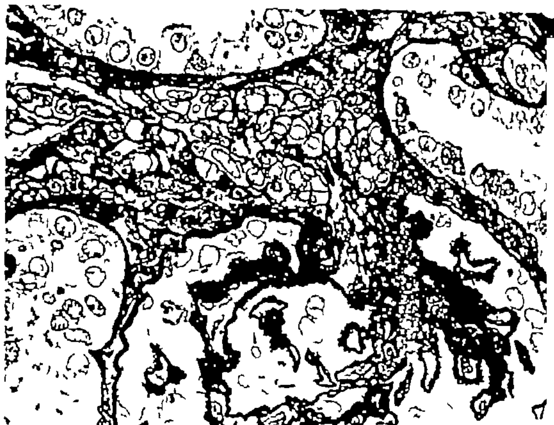


Fig 4 Case 11 A longitudinal section showing an afferent arteriole. There seems to be a transformation of the muscle cells in the afferent arteriole into large, clear epitheloid cells. These cells are not only located in the wall of the afferent arteriole near its junction with the glomerular tuft, but also in the wall away from the glomerulus. Perimembranous glomerulonephritis stage 2. PASM, × 500.

reduction of the "effective" blood volume (*Muller & Manning* 1963) Another problem in the studies of the renin system and nephrotic syndrome has been the differing classification of glomerulonephritis.

Thus *Massani et al.* (1966) found significantly higher angiotensin blood levels (ABL) in 5 cases with nephrotic syndrome (2 of the patients suffering from "membranous glomerulonephritis") whereas in "chronic glomerulonephritis" the ABL did not differ significantly from the control values. *Veyrat et al.* (1964) studied 6 patients with generalized oedema (three of them with nephrotic syndrome) Five of these showed a significant increase in the arterial renin activity *Turgeon & Sommers* (1961) studied 10 cases of "subacute and chronic" glomerulonephritis and found a markedly elevated Juxtaglomerular Cell Count. Of special interest is their observation that the hyperplasia was more marked in early stages of the glomerulonephritis obviously independent of the level of blood pressure.

As could be expected from a theoretical point of view we found a statistically significant increase in the size of the JGC-complexes in our 23 cases of perimembranous glomerulonephritis (Fig 7) A correlation between the size of the JGC-complex and the morphological stages of PGN was found so far as the JGC-complex is significantly enlarged in stages 3 and 4 ($p < 0.005$) It should be noted that the mean serum creatinine level of 5.0 mg/100 ml in our cases of hypertension is significantly higher than that in the group of normotensive patients where it was 1.5 mg/100 ml serum creatinine. Exp-

cally we found no correlation between the JGC-complex and the proteinaemia.

In perimembranous glomerulonephritis there seems to be a chronic stimulation of the JGC-complex. This is confirmed by our findings of a significantly increased mean size of the JGC-complex in this disease ($\bar{x} \pm$ S.D = $1072 \pm 148\ \mu$ as compared with $919 \pm 54\ \mu^2$ in the control group, Fig. 2) From a morphological point of view the hyperactivity of the JGC-complex in perimembranous glomerulonephritis seems to be based on an increased number of epithelioid cells in the wall of the afferent arteriole (Fig 4) and a hyperplasia and an apparent transformation of the Goormaghtigh cells into larger cells of epithelioid like appearance (Fig. 1)

The almost equal size of the JGC-complex in the normotensive and hypertensive group of PGN (1089 μ as compared with 1067 μ) is of interest as hypertension is known to exert a suppressive effect on the JGA, confirmed by the atrophy of the JGA found in the contralateral kidney of hypertensive unilateral renal artery stenosis and in primary aldosteronism (Conn-syndrome) Until today our knowledge concerning the RAAS in nephrotic syndrome is insufficient. Therefore we cannot explain why the raised blood pressure does not produce a reduction in the size of the JGC-complex in our group of hypertensive patients.

The lowering of the glomerular filtration rate (GFR) in the advanced stages of perimembranous glomerulonephritis, indicated by the higher creatinine levels in the hypertensive patients, could contribute to electrolyte and water disturbances. In addition to the presumable secondary aldosteronism, we propose that this could lead to an increased reactivity of the vasculature to angiotensin II thus giving rise to hypertension.

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POSTMORTEM FINDINGS IN PRIMARY FAMILIAL AMYLOIDOSIS WITH POLYNEUROPATHY

A Study Based on Six Cases from Northern Sweden

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The pathology of primary familial amyloidosis with polyneuropathy is described on the basis of post-mortem examination of six cases from Northern Sweden. Clinically the disease is characterized by progressive sensory and motor disturbances with loss of sensation, muscular wasting and flaccid paralysis. Impotence, urinary bladder dysfunction, motility disturbances of the gastro-intestinal tract and postural hypotension indicate affection of the autonomic nervous system as well. Malabsorption, cardiac insufficiency and vitreous opacities also occur. As regards the distribution of amyloid, the following findings seemed to be characteristic. Usually there were no gross lesions indicating the amyloid disease. Histopathologically, amyloid deposits were observed in great extent in the peripheral nervous system and in various parts of the peripheral autonomic nervous system as well. It occurred extensively in the walls of blood vessels of various calibres, in the perivascular collagenous connective tissue and adjacent to the smooth musculature. Amyloid deposition was also found more or less abundantly in various other organs and tissues. No deposits, however, or only insignificant amounts, were found in the central nervous system, either in the parenchyma of the liver, in the islets of Langerhans, or in the bone marrow. Clinical manifestations seemed to be related to the local deposition of the amyloid substance. Our clinical and pathological findings in this particular type of familial amyloidosis conformed mostly to those previously described.

Key words: Familial amyloidosis; polyneuropathy; postmortem findings.

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About 25 years ago *Andrade* described me cases of primary amyloidosis with poly uropathy which had appeared in Portugal. n that report, the disorder was recognized as separate disease entity, and the familial oc urrence of the disease was pointed out for e first time (*Andrade* 1952). Later on, several additional familial cases of neuropathic amyloidosis have been reported from various countries. The mode of inheritance is interpreted to be autosomal dominant (*Andrade* 1952). As the clinical pattern may differ to a greater or lesser degree from that of the tuguese cases, it has been suggested (*Ar et al.* 1970, *Mahloudji et al.* 1969)

disease primary familial amyloidosis with polyneuropathy might be subdivided into 3 types

(I) The Portuguese type in which the symptoms and signs of peripheral neuropathy primarily involve the lower extremities. This type is characteristic of the disease in Portuguese families (*Andrade* 1952) and of cases of Portuguese descent in other countries (*Andrade et al.* 1970) it is characteristic also of the disease in, e.g. Japanese (*Araki et al.* 1968) and some North American families (*Kantarjian & DeJong* 1953 *Kaufman & Thomas* 1959) and in one German family (*Delank et al.* 1965)

(II) The Indiana Maryland type with onset of the neuropathy in the upper extremities. This is typical of the disease in North American families in Indiana and Maryland (*Mahloudji et al.* 1969 *Rukavina et al.* 1956 *Sallman et al.* 1960 *Schlesinger et al.* 1962) Cases of this type have also been reported from Switzerland (*Klein et al.* 1962)

(III) The Iowa type. In this type, reported from Iowa, USA, the neuropathy primarily involving the legs, is combined with nephropathy and peptic ulcer (*Van Allen et al.* 1969)

An apparently different disease entity of familial systemic amyloidosis with nervous involvement has been reported from Finland (*Meretoja* 1969)

From Portugal the findings obtained at 10 complete autopsies have been reported (*Silva Horta* 19.5 *Silva Horta et al.* 1964 *Silva Horta & Trincao* 1963) Post mortem findings in a few cases of type I have also been reported from other countries (*Delank et al.* 1965 *Kantarjian & DeJong* 1953 *Kaufman & Thomas* 1959) as well as findings in type II (*Klein et al.* 1962, *Mahloudji et al.* 1969 *Rukavina et al.* 1956) and type III (*Van Allen et al.* 1969) Most of these other accounts are only brief however often including descriptions of just a few organs.

Since 1965 about 60 cases of amyloidosis with polyneuropathy most of them familial, have been diagnosed in Northern Sweden (*Andersson* 1970 *Andersson & Kassman* 1968) Clinically there seem to be many similarities between the Swedish and Portuguese cases. In our cases, the disease seems to be inherited in an autosomal dominant manner too but the penetrance is reduced and the expressivity rather variable (*Andersson* 1975)

Complete autopsies have been performed on 4 of our 60 cases and some organs from 2 additional cases have been examined post mortem. These 6 cases form the basis of the present report. The findings obtained at one of these complete autopsies have been given in a preliminary report (*Hofer & Andersson* 1969)

The present study had two main purposes to analyse in detail the distribution of amyloid in various organs in our Swedish cases and to compare our findings with those obtained in other families with neuropathic amyloidosis of type I in particular with those reported from Portugal. The resulting question about appropriate biopsy procedures to be used for diagnostic purposes in this form of amyloidosis has been discussed in a separate report (*Andersson & Hofer* 1974 b)

PATIENTS

The six cases comprised in this study had all been admitted to the Department of Internal Medicine of the University Hospital in Umeå because of serious manifestations of their disease. The clinical pattern was characterized by polyneuropathy (progressive sensory and motor disturbances with loss of sensation, muscular wasting and flaccid paresis) had commenced distally in the extremities. In all cases, the symptoms had appeared first and most markedly in the lower limbs. Various manifestations, such as impotence urinary bladder dysfunction, motility disturbances of the gastro-intestinal tract and postural hypotension, indicated affection of the autonomic nervous system as well. Malabsorption, signs of heart affection and opacities of the vitreous body were also observed. Details of the clinical findings are described elsewhere (*Andersson* 1970, *Andersson & Bjerle* 1975 a, 1975 b, *Andersson & Blom* 1972, *Andersson & Hofer* 1974 a, *Andersson & Kassman* 1968)

In accordance with these other reports, each case in this study is recorded with a capital letter indicating a family followed by a figure. In 5 cases (A 2 B 1 B 3 E 1 G 1) the diagnosis of amyloidosis had been established before death by

TABLE 1 *Clinical Data Concerning the 6 Cases of Primary Familial Amyloidosis with Polyneuropathy Forming the Basis of This Report*

Case	Sex	Age at death	Duration of symptoms (years)	Clinical comments	Immediate cause of death
A 2	F	68	15	Polyneuropathy	Circulatory insufficiency
A 7	M	68	18	Polyneuropathy Malabsorption Heart failure	Circulatory insufficiency
B 1	F	61	5	Polyneuropathy Malabsorption Cachexia	Pulmonary embolism Bronchopneumonia
B 3	M	46	8	Polyneuropathy Malabsorption Nephrotic syndrome	Circulatory insufficiency Uraemia
E 1	F	66	12	Polyneuropathy Stokes-Adams attacks (pacemaker)	Cerebral thrombosis
G 1	M	60	5	Polyneuropathy	Peritonitis. Perforation of small intestine

amination of various biopsy specimens. Information concerning age at death, immediate cause of death and duration from onset of clinical symptoms are given in Table 1

METHODS

Four complete autopsies (A 2 B 1 E 1 G 1) were performed at the Department of Pathology Umeå. Tissue specimens were taken from most of the internal organs. In two cases (A 7 and B 3) where autopsies were performed at other hospitals, tissue specimens, fixed in formalin, were sent to the Department of Pathology Umeå.

The specimens were fixed in 10 per cent formalin, dehydrated cleared, and embedded in paraffin. The cut sections were, according to routine, stained with van Gieson's stain or with haematoxylin and eosin. The amyloid stains used were alkaline Congo red (*Puchtler et al.* 1962) and in one case also Thioflavine T (*Vassar & Culling* 1959) Sections stained with alkaline Congo red were examined both in transmitted illumination and in polarized light (*Missmahl & Hartwig* 1953) Sections stained with Thioflavine T were examined in a fluorescence microscope with an Osram HBO mercury lamp equipped with exciter filter (Zeiss, Sp-orange and Sp-gelb) The periodic acid-Schiff (PAS) technique was performed on tissue specimens from case G 1 and on sections from one eye of case B 1 The myelin sheaths of peripheral nerves were examined after staining with Sudan IV and Sudan Black B, dissolved in propylene glycol (*Chiffelle & Putt* 1951) The copper phthalocyanin method /Luxol Fast blue/ (*Pearse* 1968) as well as other lipid histochemical methods (*Adams* 1965) were also used in studies of the myelin sheaths

ILLUSTRATIVE CASE HISTORY OF ONE PATIENT

As details of the clinical findings in these patients are described elsewhere (cf *Andersson* 1975) it was considered to be sufficient here to exemplify the clinical manifestations and the clinical course by giving the case history of one illustrative patient only

Case B 1 Woman born in 1906. She had had pneumonia in 1936 Because of ovarian fibromyoma, bilateral salpingo-oophorectomia was performed in 1963 Cholecystectomia was performed in 1964 because of cholelithiasis.

Symptoms of neuropathy appeared in 1962. She had piercing, burning pains in the feet and calves. Later she complained of a cushion-like sensation under her feet when walking, and the temperature-sensibility of her feet was disturbed. Examinations at that time revealed signs of incipient neuropathy in the legs. Some wasting of the lower limbs was

found. Later on, symptoms also appeared in her hands and arms. There was progression of the symptoms in all limbs with increasing wasting of the muscles and reduced sensitivity. She also had marked disturbance of the intestinal function with frequent attacks of diarrhoea. The function of the anal sphincter was impaired. In 1966 disturbance of the vision of the right eye appeared. She complained of dark, moving threads in the field of vision.

The patient was admitted to the Department of Internal Medicine of the University Hospital in Umeå in 1966. She was found to be almost cachectic. Her voice was rather hoarse. The skin was dry with small, trophic ulcers on her fingers and feet. Physical examination of the heart and lungs revealed nothing abnormal. The blood pressure was 110 mm Hg systolic and 80 mm Hg diastolic. There was no lymphadenopathy. The liver and spleen could not be palpated. Further examination revealed signs of marked polyneuropathy. She had total loss of all sensitivity of the legs, up to the groin, and of the hands. She also had extensive atrophy and flaccid paralysis in the distal parts of the limbs. She could stand up and walk only with assistance. There was general areflexia of the limbs. In the right eye, floating, band-like opacities occurred in the corpus vitreum. Electromyographic examination showed total denervation in the feet. Laboratory investigation revealed signs of intestinal malabsorption. Amyloid deposits were found at histopathological examination of biopsy specimens from the sural nerve, skin and rectum.

A family history taken at that time, revealed that two cousins of the patient and a son of one of these also had symptoms and signs of the same character. Subsequent studies proved that amyloidosis was present in these and in other members of the family as well (*Andersson* 1970).

The patient's condition deteriorated further. Her peripheral neuropathy progressed, as did the gastro-intestinal dysfunction including obstinate diarrhoea and incontinence. Vitreous opacities also appeared in the left eye, and later on keratitis and corneal ulcers. She died in 1968. The immediate cause of death was bronchopneumonia and pulmonary embolism.

RESULTS

Gross Findings at Autopsy

Detailed descriptions concerning the macroscopical findings at autopsy on 4 cases (A 2, B 1, E 1, C 1) were available. In case B 1 patches of the right atrial wall of the heart were 4 mm thick and had a firmer consistency and a more yellowish hue than the rest of the organ. Otherwise no gross lesions indicating the presence of amyloid deposition were found in these 4 cases.

All four cases had atrophy of the skeletal musculature. This was most marked distally in the extremities, especially in the lower limbs. Case B 1 was almost cachectic and in case A 2, a marked loss of subcutaneous fat was also found. Other gross findings worthy of note are given below for each case.

Case B 1 Small areas of petechiae were seen on the right breast, below the right costal margin, at the extensor surface of the right forearm and in the groin. There were small superficial ulcers on both ears and fissures in the angles of the mouth. Oedema-like lesions at low sites on the legs and the back of the feet were in evidence, whereas the skin surface in the same locations was dry and fissured. Bedsores occurred on each hip. The left pleural cavity contained 900 ml of a clear straw-coloured fluid, the right cavity containing more than 1.000 ml of a reddish-brown, somewhat opalescent fluid. In the right side there were thin fibrous adhesions. The heart weighed only 170 g. There were small amounts of thrombotic material in the right auricle. The coronary vessels showed moderate atherosclerosis. Bilateral femoral vein thrombosis was observed and, in the right main branches of the pulmonary artery a thromboembolus was found branching to one vessel in each lobe of the right lung. In larger and medium-sized bronchi there was abundant and partly purulent mucus. In all pulmonary lobes pus could be squeezed from bronchioles. The left lower lobe was atelectatic and smaller portions of atelectatic tissue were found in the right lower lobe. In the right middle lobe there was a walnut-sized red infarct. The spleen weighed 110 g. It was flabby and from the cut surface moderate amounts of soft tissue were fairly easily scraped off with the knife. The right kidney weighed 90 g and the left 80 g. In the right kidney there was a small [illegible] thrombus. The liver weighed only 940 g and the total weight of the adrenal glands did not exceed 14 g.

Case A 2 The lungs and the liver showed signs of a relatively acute circulatory insufficiency. The heart was, however, of normal size (350 g) and there was no infarct or coronary occlusion.

Case E 1 The heart, weighing 620 g, showed a pronounced hypertrophy, particularly of the wall of the left ventricle. In the apical part of the left ventricle, the wall was, within a 5 cm wide area, thin and fibrotic, apparently constituting the area of a healed infarct. In the right ventricle the electrode of an artificial pacemarker had been inserted. There was only a slight coronary atherosclerosis. Patches and confluent areas of bilateral bronchopneumonia were seen. The liver showed

signs of acute circulatory insufficiency. The left middle cerebral artery was totally occluded by a thrombus more than 1 cm long. In the left cerebral hemisphere there was a relatively fresh encephalomalacia, centrally located, measuring 5 × 5 × 3 cm. Other findings were red bone marrow in the femoral diaphysis, colonic diverticula, an almond-sized submucous lipoma in the proximal part of the colon, and a pea-sized sessile polyp in the distal part of the colon.

Case G 1. There were some scars and crusts, mainly located in the skin of the knees, representing remnants after earlier bullous lesions. There was fluid, seemingly containing small amounts of blood, in the pleural cavities, 550 ml in the right and 250 ml in the left. In the pericardial sac the amount of fluid was not increased but here, too, it seemed to contain small amounts of blood. In the peritoneal cavity there was 1 100 ml purulent fluid. The heart, weighing 550 g, showed general enlargement, especially of the left ventricle. The fact that the cut surface of the myocardium may have been more yellowish than normal could have passed unnoticed, had not the amyloid diagnosis been known before death. There was a moderate coronary atherosclerosis. The lungs and the liver showed signs of acute circulatory insufficiency. In the middle part of the small intestine there was a stricture with concomitant hyperaemia and oedema of the wall. The content of the intestines, beyond the stricture, was slightly haemorrhagic; the stricture could represent a healed perforation. The kidneys weighed together 29 g.

Histopathological Findings

It was found that the basic pattern was essentially the same in all 6 cases examined in the present study. Accordingly the histopathological findings in one typical patient only are described in detail below, namely case B 1. The occurrence of amyloid deposits in organs from all patients is summarized in Table 2.

Case B 1. Tissue specimens from this patient were examined after staining with alkaline Congo red and Thioflavine T, respectively (see Methods). The findings of amyloid deposits were the same whether one or the other method was used.

Circulatory system. Nodular amyloid deposits, in some areas forming continuous bands, were found in the endocardium (Fig. 1). Deposits were also located in the valves. In sections from the septal band, amyloid was detected around some Purkinje fibres. The myocardium showed partly nodular deposits, especially subendocardially, and partly filiform deposits, often indicating the boundaries of muscle cells. Otherwise, there was a slight to moderate interstitial fibrosis in the myocardium. In the subepicardial adipose tissue, amyloid was found in vessel walls and in small amounts in a few nerves. In the aorta, the substance appeared in connective and adipose tissue, in nerves and in vessel walls in the adventitia. Amyloid was also found in insignificant amounts in the peripheral parts of the media. The frequent presence of amyloid in the walls of blood vessels of various calibres, arteries and veins, was obvious from the examination of the individual organ systems.

Respiratory tract. Besides occurring in vessel walls, amyloid often appeared around mucous glands. In the vocal cords, deposits appeared in nerves, around muscle cells and in the collagenous connective tissue. In the bronchi and trachea, amyloid was seen adjacent to smooth muscles and in the trachea also adjacent to elastic tissue in lamina propria. In the adventitia of the trachea and of larger bronchi there were deposits in collagenous connective tissue, in nerves and vessel walls, and also in adipose tissue in the adventitia of the bronchi. In the lungs, leucocytes were observed in some of the alveoli and in a few bronchioli. The red infarct showed large amounts of a purulent haemorrhagic exudate in the alveoli. Small amounts of amyloid were seen in vessel walls in the lungs (Fig. 2) and in insignificant amounts in the alveolar walls.

Lymphoid organs. The spleen showed few filiform deposits of amyloid in the parenchyma and small deposits in a few vessel walls. In lymph nodes from the mesentery and from the lung hilus there was amyloid in blood vessels located in the surrounding tissue, in connective tissue capsules and in hilus.

Endocrine organs. In the thyroid, the adrenals and the pituitary there was amyloid only in vessel walls. Parathyroid glands showed, in addition, a few small streaky deposits interstitially. Amyloid was found in the surrounding connective tissue of the pituitary. The thyroid showed slight lesions typical of nodular goitre. The amyloid deposits in the endocrine glands caused no degenerative changes in the parenchyma. Concerning the islets of Langerhans, see below.

Digestive system. In the tongue, amyloid deposits were found in vessel walls and as strips and loops in the striated musculature. The muscular layer of the mucosa contained abundant deposits of amyloid in the stomach, duodenum, small and large intestine (Fig. 3). On the other hand, there was practically no amyloid in this structure in the oesophagus. Amyloid was found in the Brunner's glands. In the submucosa of the gastro-intestinal tract, amyloid appeared in vessel walls and in the perivascular connective tissue. The external muscular layer showed varying amounts of amyloid. Often these deposits were fairly insignificant, appearing

TABLE 2. *Occurrence of Amyloid Deposits in Autopsy Specimens from the Present 6 Cases of Primary Familial Amyloidosis with Polyneuropathy*

Organ or tissue examined	Number of cases with amyloid/ examined cases	A:2	A:7	B:1	B:3	E:1	G:1
Heart valves	2/2			+ +			++
Myocardium	3/3	+ (+)		+ +	+ +	+ +	++(+)
Aorta	3/3	+ +		+ + +			++
Lung	5/5	+ (+)		+ (+)	+ (+)	+ (+)	+(+)
Tongue	2/2	+ +		+ +			
Epiglottis	2/2	+ +		+ +			
Oesophagus	3/3			+ +		+ +	++
Stomach	4/4	+ (+)		+ +		+ +	++
Duodenum	4/4	+ (+)		+ + +	+ +		++
Small intestine	6/6	+ (+)	+ +	+ +	+ +	+ +	++
Colon	5/5	+	+ +	+ +		+ +	++
Pancreas	4/4	+ (+)		+		+ (+)	+( )
Liver*	6/6	+	+	+	+	+	+
Gall bladder	3/3	+ +		+ +			+++
Spleen	4/4	+		+ (+)		+	+
Lymph node	2/3	+ +		0			+
Tonsil	1/1	+					
Thyroid gland	4/4	+ +		+		+	+
Parathyroid glands	2/2	+ (+)		+ +			
Adrenal glands	1/4	0		0		0	(+)
Pituitary gland	2/3	+ +		0			+
Islets of Langerhans	0/3	0		0			0
Kidneys	6/6	+ (+)	+ +	+ +	+ + +	+ +	++
Urinary bladder	6/6	+ +	+ +	+ +	+ +	+ +	++
Ovarium	2/2	+ (+)		+ (+)‖			
Fallopian tubes	2/2	+ +		+ +‖			
Uterus	2/2	+ (+)		+ +			+
Testis	1/1						+
Epididymis	1/1						++
Vas deferens	1/1						+++
Prostate	2/2		+ + +				0
Brain	2/4	+†		0		+†	[illegible]
Spinal cord	2/4	+†		0		+†	++
Spinal ganglion	3/3	+ + +				+ +	+
Meninges	4/4	+		+		+	+
Choroid plexus	3/3	+		+			++
Peripheral nerves	5/5	+ + +		+ + +	+ + +	+ + +	[illegible]
Vitreous body	2/3	+ +		+ +		+ (+)	+(+)
Skin	4/4	+ +		+ + +		+ (+)	+(+)
Skeletal muscle	4/4	+		+		0	(+)
Bone marrow	2/4	+		0			

0 = no amyloid, + = essentially vascular deposits only ++ = slight to moderate deposits outside vascular structures, + + + = abundant amyloid. Brackets indicate subtle differences between the various grades. * Deposits found in the portal triad only ‖ Findings in organs extirpated 5 years before death
† Amyloid deposits suggestive of senile plaques.



Photomicrographs illustrating findings by microscopy of amyloid (black). *Fig 1* Case B:1. Nodular deposits of amyloid in the endocardium, bordering on the myocardium. *Fig 2* Case B:3. Amyloid in the walls of tw[illegible] vessels in pulmonary tissue. *Fig 3* Case B:3. Deposition of amyloid is seen in the muscular coat of the mucosa in the small intestine (arrow). *Fig 4* Case B:3. This section illustrates the presence of amyloid in the external muscular layer of the small intestine. The deposits, being rather insignificant, appear on the borderline to the submucosal layer (between arrow-heads) and to the subserosal layer (arrows). Alkaline Congo red stain. × 133



Fig 5 Case B 3 This section of the liver illustrates amyloid (black) appearing only in a vessel wall in the portal space. *Fig 6* Case B 1 Irregular lumps of amyloid in the vitreous body *The retina is seen* to the left. *Fig 7* Case B 1 This is a section of a peripheral nerve. Amyloid is seen as round deposits. Alkaline Congo red stain. × 161

in particular on the border-line to surrounding tissue (Fig. 4). The region of the Auerbach's plexus was essentially free from amyloid. In the colon as well as in the mesentery amyloid appeared in vessel walls and in nerves. There was no apparent atrophy of the villi in sections from duodenum and jejunum. In the pancreas there was amyloid in vessel walls. It was also seen in nerves in the surrounding adipose tissue, but both the acinar parenchyma and the islets of Langerhans were free from amyloid deposits. The liver showed slight to moderate fatty changes and signs of oedema. Amyloid was found only in vessel walls in the portal spaces (Fig. 5) and in the capsule.

Urogenital organs. The most abundant deposits of amyloid in the kidneys were found in the cortical-medullary boundary in vessel walls, and in the peritubular connective tissue. The glomerular deposits were usually insignificant and only rarely more moderate. The substance was found in a few arteriolar walls. Otherwise, there was a moderate interstitial fibrosis. Small accumulations of round cells were found subcapsularly. In the hilar adipose tissue amyloid occurred in nerves and vessel walls. It was also found in the ureters where the deposits appeared adjacent to smooth muscles. In the urinary bladder amyloid was seen in vessel walls, in the muscular layer and in collagenous connective tissue. Amyloid appeared in vessel walls in the ovaries and to some extent also in a fibromyoma. In the Fallopian tubes there were deposits in vessel walls and nerves of the adventitia and in vessel walls and smooth muscles of the muscular layer. In the uterus, amyloid appeared in the stroma of the endometrium, in smooth muscles of the myometrium, and in vessel walls and nerves. Amyloid was also found in the portio where it was located in vessel walls and in the collagenous connective tissue. It had a similar distribution in the vagina where it also occurred in a few nerves.

Eyes. Amyloid was found in the connective tissue around the optic nerve but not in the nerve itself. There were also deposits in vessel walls and in nerves surrounding the bulb, and further in the sclera, chorioid and retina. In the vitreous body irregular lumps of amyloid were seen (Fig. 6). The substance was found also in the perivascular connective tissue just beneath the conjunctiva. There were no lesions in the cornea or iris. In the sections of the eye a few PAS-positive cells were found e.g. in the stroma of the ciliary body.

Central and peripheral nervous system. In the meninges of the brain, amyloid was seen only in vessel walls and not in significant amounts in the surrounding connective tissue itself. In the meninges of the spinal cord, however, the substance appeared also in the connective tissue.

In the first examined sections of the brain, amyloid was seen only in superficial blood vessels. At more extensive studies, a few round corpuscles were seen in some areas. They gave faintly positive amyloid reactions and were suggestive of senile plaques. Abundant deposits were found in vessel walls in the chorioid plexus. No amyloid was seen in the pineal body.

No amyloid deposition could be found in the parenchyma of the spinal cord. A few round deposits occurred in some spinal nerve roots.

In peripheral nerves, including nerves of the autonomic nervous system, round and streaky deposits of amyloid were found (Fig. 7). In peripheral ganglia of the autonomic nervous system, however, only insignificant amounts were seen. Staining methods for myelin sheaths and other lipid histochemical procedures revealed an apparently reduced number of myelin sheaths in the femoral nerve. No degeneration of the myelin sheaths, however, and no cellular reaction was observed. The round amyloid deposits were faintly stained by Sudan Black B. The amyloid deposits in the femoral nerve appeared especially in the endo- and perineurium, to a much smaller extent in the epineurium.

Bone marrow. No amyloid was seen in the red or yellow bone marrow. There were, however, deposits in the surrounding connective tissue.

Skeletal muscles. In the skeletal musculature there was a marked, distinctly neurogenic muscular atrophy. Amyloid was found in the walls of the blood vessels.

Skin. Skin from the dorsum of one foot showed a thin epidermis with a slight, partly follicular hyperkeratosis. In the connective tissue of the corium there were lumps and streaks of amyloid, sometimes occurring distinctly perivascularly. Against the background of preceding findings in biopsy specimens, it seems likely that some of the lumps in the connective tissue represented arrector pili muscles saturated with amyloid. In the subcutaneous adipose tissue, amyloid appeared in the perivascular connective tissue.

DISCUSSION

As noted before, our Swedish cases have a clinical pattern in many respects similar to patterns observed in other previously reported cases belonging to type I. Detailed descriptions have been given from Portugal about post mortem findings in this type (*Silva Horta* 1955, *Silva Horta* & *Trincao* 1963, *Silva Horta et al.* 1964). It was natural, therefore, to compare our results particularly with those reported from Portugal, but also with those of the same type reported from other countries (*Delank et al.* 1965, *Kantar*

jian & DeJong 1953 *Kaufman & Thomas* 1959)

Gross Findings

It is apparent from our findings as well as from the Portuguese reports that gross signs, or only non-characteristic signs, indicating the presence of amyloid deposition, are not general findings in familial neuropathic amyloidosis of type I. The fact that the heart weight has been found to be increased in some patients, as observed also in our series, (*Coelho & Pimentel* 1961 *Delank et al.* 1965 *Kaufman & Thomas* 1959) may however be related to the amyloid disease. Enlargement of the spleen has also been observed in some cases (*Delank et al* 1965 *Silva Horta et al.* 1964) Petechial skin lesions, occurring fairly often in systemic amyloidosis (*Brownstein & Helwig* 1970) appeared infrequently in our patients. Bullous lesions and small ulcers of the skin, found more frequently may be related to the disease, due to the fact that similar lesions can be observed in other kinds of micro-angiopathies (*Hofer* 1973 *Hofer et al.* 1974 *Melin* 1964)

Histopathological Findings

Circulatory system. Deposits in the heart in cases of type I have been described by several authors (*Coelho & Pimentel* 1961 *Delank et al* 1965 *Kantarjian & DeJong* 1953 *Kaufman & Thomas* 1959 *Silva Horta* 1955) Nodular deposits can occur both in endo- and pericardium. Amyloid has also been found in the heart valves (*Coelho & Pimentel* 1961) Conduction disturbances occur frequently which, according to some authors, indicate involvement of the specific tissues (*Coelho & Pimentel* 1961) In case B 1 we found amyloid deposition adjacent to Purkinje fibres in the heart. It may also be pointed out here that patient E 1 had complete atrioventricular block and Stokes-Adams attacks during the final years of her life. This necessitated treatment with a pacemaker In agreement with our findings, the most abundant deposits in the myocardium have been found in the subendocardial regions (*Coelho & Pimentel* 1961) In addition, amyloid i[s] accumulated as more or less extensive rin[gs] around the muscle fibres (*Coelho & Pimentel* 1961 *Silva Horta et al.* 1964)

In the aorta, amyloid has been demonstrated in vessels and nerves of the wall, more rarely in the media and intima (*Silva Horta* 1955) As in the Portuguese cases (*Silva Horta et al.* 1964) we found that amyloid might appear in blood vessels of various calibres.

On the whole, it seems obvious that the lesions in the cardiovascular system in patients comprised in our series have a distrib[u]tion similar to that in other cases of type [I] especially those reported from Portugal.

Respiratory organs Deposits have been [re]ported to occur in the smooth musculature [of] the Eustachian tube (*Silva Horta et al.* 196[4]) The findings in our patient B 1 indicate th[at] the hoarseness which is a fairly common symptom in these patients may be caused [by] amyloid infiltration in the nerves and musc[u]lature of the vocal cords. In the lungs de[]posits are confined to vessel walls (*Kantarjian & DeJong* 1953 *Silva Horta* 1955) or the[re] are none at all (*Delank et al.* 1965)

Digestive organs. The presence of amyloid in the tongue (*Silva Horta* 1955) salivar[y] gland (*Delank et al.* 1965) and in the oeso[]phagus (*Silva Horta* 1955) is only brief[ly] mentioned in earlier reports on type I. Bo[th] our findings and those reported by other[s] (*Delank et al* 1965 *Kantarjian & DeJong* 1953 *Silva Horta et al.* 1964) indicate tha[t] the histopathological lesions in the gastro[-]intestinal tract can vary within wide limi[ts] from case to case Through different types [of] studies it has been concluded that the gastro[-]intestinal disturbances to appear in this di[s]ease are mainly secondary to lesions in the autonomic nervous system (*Monteiro* 196[.] *Rosario et al* 1963) Contrary to *Silva Horta* (1965) we found in our cases only insignifi[-]cant deposition of amyloid, or none at all, i[n] the region of Auerbach's plexus. However, deposits in the nerves were observed in the serosa and in the mesentery

Varying amounts of amyloid can occur i[n]

pancreas in type I (*Kantarjian & DeJong* 1953 *Silva Horta* 1955). In our cases, examined in this respect, there were no deposits of amyloid in the islets of Langerhans. Even in advanced cases with atrophy and disappearance of parenchyma, the islets of Langerhans seem to be spared (*Heller et al.* 1964). This is quite opposite to the findings in amyloidosis of the aged where these islets seem to be affected almost exclusively (*Schwartz et al.* 1964 *Westermark* 1972).

With one exception (Delank et al. 1965) amyloid has usually been found in the liver. In agreement with our findings it has been observed that the amyloid deposition in this type of familial neuropathic amyloidosis, does not to occur outside the portal triad (*Silva Horta et al.* 1964).

Lymphoid organs. Varying amounts of amyloid have been reported to occur in the spleen (*Delank et al.* 1965 *Kantarjian & DeJong* 1953 *Silva Horta* 1955). As regards the patients in our series in whom amyloid was present in the spleen, the deposits mainly appeared in walls of arteries of various size. Thus, the occurrence of small deposits in the red pulp was exceptional. In lymph nodes, the deposition can be confined to the capsule and the surrounding adipose tissue (*Silva Horta* 1955). Parenchymal lesions, however may also occur (*Delank et al.* 1965). In the tonsils, amyloid has been demonstrated in vessel walls and nerves. These observations agree with those described in the present report.

Endocrine organs. In these organs, the deposition of amyloid can also vary from vascular deposits only to more extensive deposition in the parenchyma. Amyloid has been reported to occur in the thyroid (*Delank et al.* 1965 *Silva Horta* 1955) parathyroid (*Silva Horta* 1955) adrenals and the pituitary (*Delank et al.* 1965 *Silva Horta & Trincao* 1963). The statements in these reports also agree with our findings. The islets of Langerhans are discussed under the heading of digestive organs"

Urogenital organs. Our observations concerning lesions of the urinary tract have been reported separately (*Andersson & Hofer* 1974 a). Deposits in ovaries (*Kantarjian & DeJong* 1953 *Silva Horta & Trincao* 1963) and the uterus (*Kantarjian & DeJong* 1953) have been described previously. The present report gives some additional information about the distribution of amyloid in the Fallopian tubes and in the vagina. Our findings and those reported from Portugal (*Silva Horta* 1955) indicate that, in this type of amyloidosis, the deposits in testis appear predominantly in vessel walls. In the seminal vesicles and in the prostate the deposits appear in the smooth musculature (Silva Horta & Trincao 1963).

Eye. Post mortem findings of a character similar to those in our patients have been observed in an American case of type I (*Paton & Duke* 1968). It may be added here that we found no amyloid in the optic nerve. Neither has amyloid been demonstrated in the optic tract (*Silva Horta & Trincao* 1963).

Central nervous system. Amyloid in the meninges may occur in small amounts, as in our cases, or can be abundant (*Delank et al.* 1965 *Silva Horta & Trincao* 1963). The parenchyma of the brain and spinal cord are essentially free from amyloid, a fact which from the pathogenetic point of view has been related to the blood-brain barrier (*Krücke* 1963). The substance can, however appear in subependymal regions, in the chorioidal plexuses and in the cortex as senile plaques (*Silva Horta & Trincao 1963*) as was observed also in our cases. In the brain tissue there may be a proliferation of neuroglia adjacent to the deposits in the pia mater and in the subependymal regions.

Peripheral nerves. In general the findings in our cases were of the same character as those in other cases of this type of amyloidosis (*Silva Horta & Trincao* 1963). In accordance with the clinical manifestations we found extensive deposits of amyloid in peripheral nerves, e.g. the femoral nerves. The amyloid was present as rounded or streaky deposits especially in the endo- and perineurium, to a lesser extent in the epineurium. The nerve fibres passed these deposits in an

arched manner or ended abruptly at their periphery Destructions of nerve fibres and of myelin sheaths have been observed (*Andrade* 1952 *Delank et al* 1965 *Silva Horta* 1955) This is in agreement with our findings. We also found amyloid deposition in spinal nerve roots and in spinal ganglia. The amyloid substance was likewise abundantly distributed in various parts of the autonomic nervous system. The deposits were found in nerves of most of the internal organs. Thus, deposits were present in the nerves of the heart, the aorta and blood vessels of various calibres. Also the nerves of the larynx and respiratory tract, the esophagus, stomach, intestines, pancreas, *mesenterium and genitourinary system* were affected as well as nerves of the eye. (Concerning the optic nerve see above.)

Bone marrow Amyloid has been found in blood vessels in the bone marrow in previously examined cases (*Silva Horta* 1955) As shown in some of our cases, the occurrence of amyloid in bone marrow may be related to the size of blood vessels appearing in the sections. A typical feature seems to be that amyloid does not occur outside the vessel walls.

Skeletal muscles and joints In the striated musculature in the Portuguese cases, amyloid appeared mainly in vessel walls and in nerves (*Silva Horta* & *Trincao* 1963) This is in agreement with our findings. Only occasionally do deposits occur in the sheaths of the sarcolemma. In the German case, however there were nodular amyloid deposits in skeletal musculature, in the diaphragm and tongue musculature (*Delank et al.* 1965) As in our cases, atrophy of striated muscles has been demonstrated (*Silva Horta* & *Trincao* 1963) Small amounts of amyloid have been found in the tissue of a joint capsule (*Silva Horta* 1955)

Skin. From Portugal it has been reported that the amyloid deposits in the skin are located in vessel walls and nerves, in the arrector pili muscles and adjacent to sweat glands (*Silva Horta et al.* 1964) Amyloid was found as hoops in the subcutaneous tissue and also surrounding the fat cells, particular-ly in the border line area to the derma. T findings were of the same character in a cases.

GENERAL COMMENTS

The results obtained in our study indic that, histopathologically the findings in o Swedish cases agree largely with those o served in other families afflicted with a di ease which clinically is of the same charact Detailed reports on autopsy findings a available from Portugal. Some difference the descriptions of the findings may be o incidental. We did not, for instance, find a significant deposition of amyloid in the regi of plexus Auerbachi of the intestine, which reported to be a common finding in t Portuguese cases. Findings of amyloid sub stance in the vitreous body as in two of o cases, have not, apparently been reporte from Portugal.

Regarding the other forms of famili neuropathic amyloidosis stated above, typ II and III, the reports about post morte findings are brief and comprise single ca only (*Klein et al.* 1962, *Mahloudji et a* 1969 *Rukavina et al.* 1956, *Sellman et a* 1960 *Van Allen et al.* 1969) In these type amyloid is also generally deposited in bloo vessels, connective tissue and various orga in a manner which in many respects seem t be similar to the distribution in type I. Th involvement of solid viscera, including th liver however may be more extreme in th cases of type III reported from Iowa (*Va Allen et al.* 1969)

General Features of the Distribution of Amyloid

According to *Missmahl* (cf. *Heller et a* 1964) the different types of amyloid disea

* Some biopsy specimens from our cases wi neuropathic amyloidosis have been examined b Dr H. P Missmahl Professor of Medicine a the University of Tubingen, Federal Republic o Germany He has confirmed that they all sho a pericollagenous type of amyloidosis. We ar grateful to Professor Missmahl for his kind an generous help.

y be separated histopathologically into two in groups, viz. perireticular and pericollagenous amyloidosis. The difference between the two types can most easily be recognized by studying the initial amyloid deposition in blood vessels. The Portuguese type of familial neuropathic amyloidosis has been described as belonging to the pericollagenous group (*Gafni et al.* 1964 *Silva Horta et al.* 1964). In our cases the distribution of amyloid was found to be the same.*

The observation that the amyloid deposits in this neuropathic type of amyloidosis have a tendency to be located at the borderline between different types of tissues (*Silva Horta et al.* 1964) is in agreement with our findings.

According to the biphasic cellular theory concerning the production of amyloid (*Teilum* 1969) there is an initial phase with proliferation of plasma cells and a secondary phase with amyloid deposited in situ as a production of PAS-positive reticulo-endothelial cells. In some cases of hereditary neuropathic amyloidosis, the number of plasma cells has been found to be increased in the spleen and lymph nodes (*Andrade* 1968) and in the bone marrow (*Silva Horta et al.* 1964). PAS-positive cells were observed in the vicinity of the amyloid substance in peripheral nerves (*Andrade* 1968). These observations, however, seem to be difficult to evaluate with regard to the pathogenesis of amyloidosis. In our study including detailed histological examinations of various organs and tissues, no significant increase of plasma cells was observed. Neither were amyloid deposits found to be located in any obvious proximity to PAS-positive cells.

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THE EFFECT OF TEMPERATURE ON LONG TERM CELL ADHESION

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The effect of temperature on the development and maintenance of long-term cell adhesion was studied. Cell metabolism appeared to be essential for the maintenance of long-term cell adhesion at 37 C. The maintenance of cell adhesion at low temperatures, however was less dependent on cell metabolism.

Key words cell adhesion temperature.

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In an earlier study (*Attramadal & Jonsen* 1974) the initial cell adhesion was found to increase when the temperature was increased from 4 C to 37 C. The initial cell adhesion also took place, though to a lesser extent, when cell metabolism was arrested.

The initial adhesion occurs immediately upon cell contact and should be distinguished from the subsequent long-time adhesion in which an increased strength is observed as it develops. This development is probably determined by cell phenomena such as cell motility surface deformability and surface activity in addition to synthesis of extracellular materials and specific surface structures such as desmosomes.

The present study was undertaken to study the effect of temperature on the kinetics of cell adhesion during the first hours after the initial contact is established.

MATERIALS AND METHODS

Cells and media. P 388 cells (mouse lymphoblastoma cells) were propagated as stock cultures as described earlier (*Attramadal & Jonsen* 1974) In the adhesion studies, cell samples were centrifuged and transferred to Eagle's minimum essential medium (*Eagle* 1959) with Hanks' balanced salt solution (*Hanks & Wallace* 1949) The medium was added 10,000 I U penicillin and 10 mg streptomycin (Glaxo laboratories, Greenford, England) and 10 ml foetal bovine serum (Grand Island Biological Company New York, U.S.A.) per 100 ml. The pH was adjusted to 7 4 with a 1 4 per cent $NaHCO_3$ solution.

Cell adhesion assay. Samples of 10 ml medium containing 4 10^6 cells were equilibrated to the desired test temperature and added to Carrel flasks with a diameter of 60 mm (Pyrex" J A. Jobling & Co., Ltd.) At intervals, one flask was removed and exposed to a standard agitation procedure in horizontally gyratory shaker (Thomas rotating apparatus, Arthur H. Thomas Co., Philadelphia) In this procedure the flasks were shaken for 2 minutes in a circular movement with 9 mm radius, 180 cycles per minute. As this agitation was more violent than any manipulation of the flasks during the experiments, errors caused by dissimilar handling of the flasks were largely eli-

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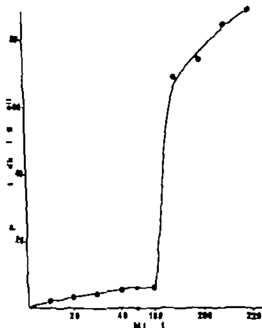


Fig. 3 The development of cell adhesion in a cell culture which was kept at 0 C for 3 hours and thereafter placed at 37 C. Each plot is the mean of two observations.

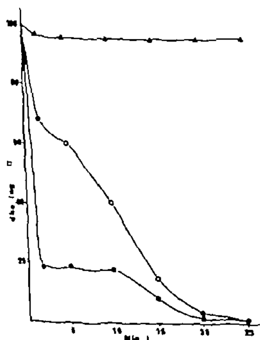


Fig. 4 The time-course of cell detachment at various temperatures in medium deficient of divalent cations. Each plot is the mean of two observations.

● 37° C ○ 22° C △ 0° C

the adhesion rate if transferred to 37 C (Fig. 3)

No adhesion developed when cell metabolism was inhibited by addition of 0.5 mM 2,4-dinitrophenol and 1 mM iodoacetate for 20 minutes.

Maintenance of cell adhesion If dinitrophenol and iodoacetate were added to cell cultures grown on Carrel flasks for 24 hours, 97 per cent of the cells would be detached within one hour.

The cell layers of 24 hours old cultures in Carrel flasks were gently flushed with 10 ml Hanks balanced salt solution without Ca and Mg added, and then added 10 ml of the same solution. The rate of cell detachment was then determined after various time intervals. The experiment was performed at 37 C, 22 C and 0 C. The detachment at 37 C occurred essentially within the first two minutes (Fig. 4). At 22 C, the detachment was slower but reached nearly 100 per cent after 20 minutes. At 0 C little detachment occurred.

DISCUSSION

Temperature changes modify the initial adhesion of cells to a glass surface (*Attramadal & Jonsen* 1974). The temperature effect on the initial adhesion is primarily thought to be a direct physico-chemical effect on the adhesion mechanism as an arrested cellular metabolism does not prevent the establishment of initial adhesion (*Attramadal & Jonsen* 1974).

The method by which adhesion has been assayed in the present study was based on a detachment technique in which cells adhering weakly to the substratum were dislodged. By the standard shaking procedure, fluid velocities of an order of 1000 cm per minute were created in the Carrel flasks, and the cells were thus exposed to shear forces far in excess of the initial cell adhesion force (*Attramadal & Jonsen* 1970). Accordingly the method is thought to measure the stronger cell adhesion which appears secondary to the initial adhesion.

The present study indicates that the development of long term adhesion is dependent on the temperature. This is in accordance with earlier reports (*Moscona* 1961 *Roth* 1968, *Jones* & *Morrison* 1969). Several authors have reported that cell to cell adhesion does not develop at temperatures below 15 C in aggregation experiments (*Moscona* 1961 *Steinberg* 1962, *Weiss* 1963). Few studies of the effect of temperature in cell to glass adhesion have been performed, but *Weiss* (1964) found cell to glass adhesion to occur at temperatures below 15 C. The present study indicates a development of long term cell adhesion down to 5 C. Fig 2 demonstrates that, while long term adhesion develops after 90 minutes at all temperatures above 0 C, it is not observed during shorter observation times until the temperature exceeds a lower limit. This lag could reflect a delay in the development of adhesion as a result of a slowed-down metabolism, but it is also possible that the development of cell adhesion below a threshold value is not recorded by the technique used.

The reversibility of the inhibiting effect of low temperature and the observation that no adhesion developed if cell metabolism was arrested, indicate that the development of long term cell adhesion is dependent on metabolic processes. *Moscona* (1961) suggested that cell adhesion was a function of the synthetic activities of the cell. *Steinberg* (1962) proposed that the surface mobility might be the temperature-dependent process, as temperature dependency was not involved in already established adhesion. In support of this view is the observation of *Michaelis* & *Dalgarno* (1971) that cells failed to spread if placed at 4 C. The present findings, however indicate that, at 37 C, not only the development, but also the maintenance of cell adhesion is dependent on cell metabolism.

The method of removing divalent cations in order to eliminate cell adhesion is well known (*Seglen 1973*). Fig 4 shows that the detachment of cells after removing the divalent cations from the medium is dependent on the temperature. It is unlikely that simple chemical effects can explain the persistence of cell adhesion at 0 C, whereas a near complete detachment is seen within 15 minutes at 37 C. It is possible however that this may be caused by physico-chemical changes in the cell surface. *Weiss* (1964) found that fewer cells could be detached from glass at low temperatures and suggested that the cell surface was stronger at lower temperatures. Likewise, *Zeidman* (1949) found by micromanipulation of attached pairs of epithelial cells that a decrease in temperature produced an increase in cell adhesion. The observation of *Mitchison* & *Swann* (1954) that the deformability of sea urchin eggs fell to one half if the temperature was lowered from 18 C to 4 C, demonstrates physico-chemical mechanisms that may be involved.

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MCG101-AA, A NEW ASCITES TUMOUR IN C_{57} MICE

4 *Influence of Enzyme Treatment on Transplantability and Some* in vitro *Characteristics*

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Hagmar Björn & Ryd, Walter MCG101-AA, A new ascites tumour in C_{57} mice. 4 Influence of enzyme treatment on transplantability and some *in vitro* characteristics. Acta path. microbiol. scand. Sect. A, 83 328–338, 1975

The transplantabilities of trypsin and neuraminidase-treated 101 AA cells were compared with untreated cells. The cell doses required for growth were similar for enzyme-treated and control cells. Yet, trypsinized cells injected i.v gave rise to less lung tumours and, in one experiment, they produced more extrapulmonary tumours than control cells or neuraminidase-treated cells. These findings were collated with some data on the effect of enzyme treatment on the tumour cell membranes, obtained by particle size analysis, cell electrophoresis, lectin agglutinability and sialic acid determinations. The importance of cell surface characteristics for specific metastasis patterns, in this case a peculiar "myotrophism" is discussed.

Key words Ascites tumour MCG101 AA transplantability mice.

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The ascites tumour MCG101 AA arose from the solid syngeneic fibrosarcoma MCG101-SS (Hagmar 1974a) The transplantabilities of the SS and AA forms were tested in comparison with the AS form, i.e. the ascites tumour growing as a solid tumour subcutaneously (*Hagmar* 1974b) The three tumour forms had different transplantabilities and in particular different "metastasis patterns" when injected intravenously This was tentatively ascribed not only to genetical changes during the ascites conversion but also to cell surface changes brought about by the enzyme treatment required to bring the SS and AS tumours into suspension.

The present study was performed to determine whether enzymatic removal of surface material may alter the transplantability of tumour cells, and their "metastasis pattern" upon intravenous injection. MCG101-AA cells were subjected to trypsin or neuraminidase treatment prior to injection into different anatomical compartments (subcutaneously, intraperitoneally and intravenously) The in vivo studies were collated with some in vitro data on the mechanism by which the enzyme treatment affected the tumour cells, particularly their surface properties.

MATERIAL AND METHODS

Tumours

MCG101-AA, serially transplanted intraperitoneally in syngeneic C 57 Bl/6J mice, was used in tran- for generations 10–40 as indicated below The

...er was harvested on day 7–10 and washed as described previously (*Hagmar* 1974a). The blood contamination necessitated hypotonic lysis in Hanks' BSS diluted 1:2 with distilled water and centrifugation (1 min. 100 g, 4 C). The procedure was repeated four times upon which the cell pellet was resuspended in Eagle's MEM. The hypotonic lysis removed more than 99 per cent of the red blood cells and in the final suspension they never represented more than 10 per cent of the tumour cell number. The loss of tumour cells in the procedure sometimes was as great as 75 per cent. The tumour cell viability by Trypan blue was 70–90 per cent before and 70–75 per cent after lysis.

...ymes Treatment

Trypsin and neuraminidase treatment of cells ...s performed on separate suspensions from the ...me pooled ascites obtained from several donors. ...he incubations were made under agitation in a ...ater bath at 37 C for 30 min. in MEM. The ...al concentrations were: 10^7 cells per ml with ...25 per cent trypsin (Difco) or ...5 U/ml neu...minidase (Behringwerke) or only MEM (con...rols). In incubations made for sialic acid deter...minations, Hanks' BSS without phenol red was used instead of MEM.

After incubation, the suspensions were centrifuged (80 g, 10 min., +4 C). The cell pellet was resuspended in MEM with DNase 60 µg/ml, recentrifuged and resuspended in medium with 30 µg/ml DNase. In the *in vivo* studies, MEM 90 per cent and syngeneic serum 10 per cent were used, otherwise only MEM. Cell losses during incubation ranged from 20 to 60 per cent in different experiments, but were quite similar in the control and in the enzyme treated suspensions.

Particle Size Analysis

The cell size distribution was determined by Celloscope counts and the aggregability by cell counts on Millipore filters as described previously (*Hagmar & Norrby* 1973).

Cell Electrophoresis

A cylindrical cell apparatus (*Bangham et al.* 1958) with constant temperature (25 C) and voltage gradient (50 V) was used. As in earlier experiments (*Hagmar* 1974a) the cells were suspended in MEM (pH 7.2 ± 0.2) and human red blood cells were used as standard pre-experimentally.

Lectin Agglutinability

The aggregation by two types of lectins, Concanavalin A (Con A) and wheat germ agglutinin (Wga) was tested as before (*Kapeller & Doljanski* 1972, *Hagmar* 1974a).

Sialic Acid Release

Sialic acid was determined by *Warren's* (1959) method on cell free supernatants obtained by filtration through a 0.22 µm Millipore filter, freeze-drying and re-solubilization in water to 10 per cent of original volume. Readings at 549 and 532 nm corrected for the presence of riboses. The calculated amounts of sialic acid showed a close correlation with standard readings on dilutions of N acetylneuraminic acid (Fluka).

In vivo Studies

Suspensions subjected to trypsin or neuraminidase treatment or control cells (*vide supra*) were serially diluted in MEM 95 per cent-syngeneic serum-5 per cent to obtain appropriate transplantation doses (Tables 4, 5 and 6). Samples from the same suspensions were taken for viability and particle size analysis as indicated above. Syngeneic mice 2–3 months old were used throughout, distribution between sexes being equal. All cell doses were given in 0.1 ml, the intravenous injections int... a tail vein.

The animals were observed daily and subcutaneous takes were determined by palpation. In the case of intraperitoneally and intravenously injected animals, the time of spontaneous death was registered and the gross tumour distribution was determined at autopsy. Lungs, hearts and livers from intravenously injected animals were prepared for serial histological sections (200 µm apart) as described by *Boeryd* (1965).

Sections were also taken from spleens, each kidney and from any doubtful tumour. Lung weights after fixation were used for comparison of the tumour mass in that organ (*Hagmar & Boeryd* 1969).

Three separate studies were performed. In a pilot-study (A) performed with transplantation generation 10 multiple cell doses were used for subcutaneous (s.c.), intraperitoneal (i.p.) and intravenous (i.v.) cell injection, 4 animals being given each dose of each suspension. The two later studies (B and C) were performed with transfer generation 28 and 39. Ten animals were given either 10^5 or 10^6 cells i.v. I.p. and/or s.c. transplantation tests were here performed to control the cellular viability.

Statistical Methods

Incidences were compared by using Chi² analysis and the number of gross extrapulmonary tumours as described previously (*Hagmar & Boeryd* 1969). Where possible the average lung weights were compared by Student' t-test and the survival times by Wilcoxon two-sample rank test.

RESULTS

Particle Size Analyses

By repeated Celloscope counts, no changes in cellular volumes caused by trypsin or neuraminidase treatment were detected. The mean particle diameter was 12.4 μm in all suspensions.

Cell and aggregate counts on Millipore filters applying to the suspensions used *in vivo* are given (Table 3). As may be seen, the trypsin suspensions contained somewhat fewer cell aggregates than the other two types of suspension which were similar in this respect. The table also give the respective cellular viabilities by Trypan blue.

Cell Electrophoresis

Fig. 1 shows a representative histogram of cell electrophoresis. The range of reduction of mean mobility, determined on 4 separate occasions, was in the case of neuraminidase treatment 8–16 per cent, in the case of trypsin treatment it was 21–31 per cent.

Lectin Agglutinability

Table 1 shows a representative result of an aggregation test. While control cells were unreactive, trypsin-treated cells were readily and reproducibly agglutinated by the two lectins. Neuraminidase-treated cells showed a weak reactivity with both agglutinins, a result which was not always reproducible.

Sialic Acid Release

Table 2 shows the amounts of sialic acid released by enzyme digestion in 4 separate experiments. Although results for individual experiments vary, the order of magnitude correlates with the respective type of enzyme treatment. The supernatants from control cells contained very little sialic acid, while neuraminidase treatment released a considerable amount of free sialic acid. Trypsin supernatants were like the controls in that they showed very little sialic acid. It should be noted, however, that Warren's method measures only free sialic acid and does not measure

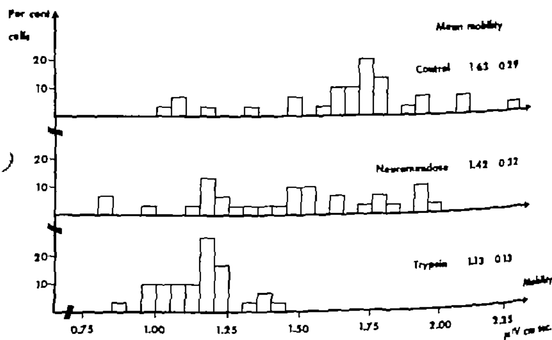


Fig. 1 Histogram of cell electrophoresis results in one representative experiment. Thirty cells were measured in each direction from all three suspensions.

TABLE 1 *Lectin Agglutinability of AA Cells with or without Enzyme Treatment*

Suspension		Agglutinin concentration					
	Con A ng/ml	400	200	100	50	25	0
	Wga	1:8	1:16	1:32	1:64	1:128	0
Control	Con A	+ +	0	0	0	0	0
	Con + MG	0					
	Wga	0	0	0	0	0	0
	Wga + NAGA	0					
Neuraminidase	Con A	+ +	+ +	+	0	0	0
	Con + MG	0					
	Wga	+ +	+ +	+ +	+	0	0
	Wga + NAGA	0					
Trypsin	Con A	+ + +	+ + +	+ + +	+ +	+	0
	Con + MG	0					
	Wga	+ + +	+ + +	+ + +	+ +	+	0
	Wga + NAGA	0					

Con A = Concanavallin A (Sigma) MG = α-metyl-D-glucopyranosid.
Wga = Wheat germ agglutinin from inactivated wheat germ lipase (Sigma)
NAGA = N-acetyl-D-glucosamine.

protein-bound which would presumably be released by trypsin. Attempts to determine the total amount of sialic acid by *Svennerholm's* (1957) resorcinol-method were unsuccessful.

In vivo Studies

In *Study A* (Table 4) the three types of suspension showed similar transplantabilities s.c. and i.p. As in a previous study (*Hagmar* 1974b) the critical cell number for growth was lower i.p. than s.c. This is true for all suspensions, although less evident for the trypsinized suspension, from which smaller cell doses grew i.p. in more mice than the 10 dose.

The survival times of i.p. injected animals showed no consistent difference between the groups. Animals injected i.v. showed no significant differences between tumour parameters in the controls and the recipients of neuraminidase-treated cells. The incidence of extrapulmonary takes tended to increase however.

In animals injected with trypsin-treated cells i.v., the lung weights were significantly smaller than in the control animals ($p < 0.05$). On the other hand, the number and incidence of extrapulmonary takes were greater in the trypsin group. However, the difference was only evident at the highest dose levels.

The metastases were similarly distributed in the three groups (not in the table). Many of the takes occurred in the heart (controls 3, neuraminidase 11, trypsin 10). More than one "take" per animal and heart was sometimes registered, i.e. when several clearly separate foci were found in the histological sections. In other organs, only grossly recognized metastases were counted. Most of these were found in striated muscle (controls 2, neuraminidase 2, trypsin 12). The remaining takes were found in lymph nodes, kidneys and fat.

In *Study B* (Table 5) the trypsin-suspension took s.c. in fewer animals than the other

TABLE 2. *Amount of Sialic Acid Released by Enzyme Treatment in 4 Separate Experiments (μmol per 2 × 10⁷ Cells)*

Experiment no.	1	2	3	4
Control cells	0.0027	0.0005	0.0012	0.0000
Neuraminidase	0.033	0.039	0.013	0.013
Trypsin	0.0059	0.0027	0.0033	0.0067

TABLE 3 *Study A-C Number of Single Cells and Aggregates per 1000 Particles on Millipore Filter Cellular Viability by Trypan Blue*

Study	Suspension	No of cells per particle								Viability in per cent
		1	2	3	4	5	6–10	11–20	>20	
	Control	869	94	10	8	2	6	8	3	80
A	Neuraminidase	838	122	18	8	3	3	3	5	66
	Trypsin	910	58	15	5	9	3	3	2	69
	Control	911	75	8	3	2	1	0	0	75
B	Neuraminidase	856	110	16	3	2	6	4	0	82
	Trypsin	905	73	16	5	1	0	0	0	83
	Control	860	113	19	4	1	0	0	0	83
C	Neuraminidase	898	71	17	7	3	3	1	0	86
	Trypsin	919	61	8	0	1	0	0	0	85

suspensions in spite of similar Trypan blue viabilities (Table 3) and similar takes-frequencies and survival times upon i.v. injection.

Again the average lung weights were smaller in animals given trypsinized cells. This time, however, there was no significant increase in extrapulmonary takes, all found in the heart or in other muscle. As above, these include gross tumours and microscopic foci in the heart. The incidence of only gross extrapulmonary takes were: controls 2/8, neuraminidase 1/10 and trypsin 6/9 ($p < 0.10$).

In *Study C* (Table 6) we used both s.c. and i.p. transplantation as viability tests. We found no differences in the incidences of takes in any of these sites, nor in the survival times. However, the i.v. injected animals given trypsinized cells lived longer than the controls ($p < 0.01$). As before, the trypsinized cells gave rise to a smaller tumour mass in the lungs than control and neuraminidase-treated cells. The controls showed a high incidence of extrapulmonary (heart) tumours: thus, the 100 per cent incidence in the trypsin-group is not significantly different. The heart metastases were often confluent and could not be adequately quantitated merely by counting. Beside the takes given in the table, 2 control mice had microscopic tumour foci in the liver.

DISCUSSION

These studies sought to determine how treatment with neuraminidase or trypsin affected the transplantability of ascites tumour cells. The first study (A) compared transplantation doses in different anatomical compartments (s.c., i.p. and i.v.). The two other studies (B and C) concentrated on the tumour patterns arising from i.v. injected cells ("experimental metastases").

In vitro methods for measuring viability (i.e. vital stains, respiratory analyses or DNA labelling) do not necessarily reflect the *in vivo* growth capacity of cells. This is better measured by transplantation tests in which cell transfer into different sites, as in our studies, safeguards against local peculiarities of growth.

As a viability control we used i.p. and s.c. transplantation in Study A, and s.c. transplantation only in Study B. When trypsinized cells showed a reduced transplantability s.c. in Study B, we reintroduced i.p. transplantation in Study C, where we did not find any evidence of trypsin damage on the transplantability.

Thus, we found no certain evidence that our enzyme treatment damaged the cells irreversibly, confirming the opinion of other researchers that the treatments adopted were harmless (*Rinaldini* 1958, *Kaatson et al* 1971, *Simmons et al* 1971, *Hauschka et al* 1971).

TABLE 4. *Study A. Incidence of Takes in Different Injection Sites (s.c., i.p. and i.v.), Mean Survival Times and Tumour Distribution from i.v. Injected Cells*

Suspension		Cell dose	s.c. Incidence	i.p. Incidence	i.p. Mean survival time (days)	i.v. Incidence	i.v. Mean survival time (days)	i.v. Lungs Incidence	i.v. Lungs Average weight ± SD	i.v. Extrapulmonary takes Incidence	i.v. Extrapulmonary takes Total no.
Control		10^4	–			3/3	25	3/3	0.42 ± 0.19	1/3	4
		10^3	4/4			4/4	23.5	4/4	0.45 ± 0.15	0/4	0
		10	2/4	4/4	27.5	3/4	51	3/4	0.41 ± 0.15	3/4	5
		10^2	1/4	4/4	27.5	4/4	40.5	4/4	0.50 ± 0.24	2/4	3
		10^3	1/4	2/4	38.5	–	–	–	–	–	
	Total		8/18	10/12		14/15		14/15	0.44 ± 0.17	6/15	12
Neuraminidase		10^4				4/4	20	4/4	0.52 ± 0.10	4/4	5
		10^3	4/4			4/4	22	4/4	0.51 ± 0.0+	2/4	4
		10	1/4	3/4	33	4/4	43.5	4/4	0.55 ± 0.35	3/4	4
		10^1	1/3	3/4	33	3/4	75	[illegible]/4	0.55 ± 0.29	3/4	5
		10^2	0/4	3/4	45	–	–	–	–	–	
	Total		6/15	9/12		15/16		14/16	0.53 ± 0.29	12/16†	18
Trypsin		10^4				4/4	22	4/4	0.37 ± 0.09	4/4	9
		10^6	4/4			4/4	52.5	3/4	0.30 ± 0.09	4/4	10
		10	2/4	1/4	19	4/4	48.5	1/4	0.34 ± 0.25	4/4	5
		10	1/4	3/4	42	3/4	93	3/4	0.30 ± 0.02	1/4	2
		10^3	1/4	3/4	29	–	–	–	–	–	
	Total		8/16	7/12		15/16		11/16	0.33 ± 0.13§	13/16§	26§

One animal discarded on account of growth at the injection site.
§ = significant difference from controls ($p<0.05$)
† = statistical tendency ($p<0.10$)

TABLE 3 *Study B. Incidence of Takes s.c. and i.v. Mean Survival Times and Tumour Distribution from i.v. Injected Cells*

Suspension		Cell dose	S.c. Incidence	I.v. Incidence	I.v. Mean survival time (days)	I.v. Lungs Incidence	I.v. Lungs Average weight ± SD	I.v. Extrapulmonary takes Incidence	I.v. Extrapulmonary takes Total no.	
Control		10^8	4/4	8/8*	23	8/8	0.48 ± 0.06	4/8	4	(heart)
		10^4	4/4	–		–				
	Total		8/8							
Neuraminidase		10^7	4/4	10/10	21.5	10/10	0.47 ± 0.13	5/10	6	(heart)
		10^4	4/4	–		–				
	Total		8/8							
Trypsin		10^8	2/4	9/9‡	25	9/9	0.35 ± 0.07†	6/9	7	(heart and other striated muscle)
		10^4	1/4	–		–				
	Total		3/8							

* Two animals of original 10 discarded on account of growth or suspected growth at injection site.
‡ One animal of original 10 discarded on account of growth or suspected growth at injection site.
† = significant difference from controls ($p < 0.05$)

TABLE 6. St dy C Incidenc of Takes c p. and i. Mean S rvival Times f i.p. and i.v. Injected Animals and Tumour Distribution in i.v. Injected Animals

Suspension		Cell dose	S.c. Inci-dence	I.p. Inci-dence	I.p. Mean survival time (days)	I.v. Inci-dence	I.v. Mean survival time (days)	I.v. Lungs Inci-dence	I.v. Lungs Average weight (±SD)	Extrapulmonary takes Inci-dence	Extrapulmonary takes Total no.
Control		10^5	–			10/10	14	10/10	0.85 ± 0.24	8/10	≥12 (heart)
		10^4	3/4	4/4	22						
		10^3	1/4	3/4	30						
		10^2	0/4	2/4	38						
	Total		4/12	9/12							
Neuraminidase		10^5	–			10/10	15	10/10	0.80 ± 0.16	5/10	≥ 6 (heart)
		10^4	4/4	4/4	26						
		10^3	1/4	2/4	30.5						
		10^2	1/4	1/4	40						
	Total		6/12	7/12							
Trypsin		10^5	–			10/10	16.5	10/10	0.64 ± 0.12*	10/10	≥15 (heart)
		10^4	2/4	4/4	29						
		10^3	1/4	4/4	27.5						
		10^2	0/4	1/4	42						
	Total		3/12	9/12							

* significant difference from controls ($p<0.05$)

Transplantation studies A and C also confirm that the 101 AA tumour still grows from smaller inocula i.p. than s.c. (*Hagmar* 1974b) remaining unaffected by either trypsin or neuraminidase treatment.

Yet, one asks, if the enzyme treatment was relatively harmless, how effective was it in modifying the cell surfaces?

Although cell volumes remained constant in all suspensions, altered agglutinability by Con A and Wga indicates an altered surface structure where new sugar groups became available, especially on trypsinized cells. Cell electrophoresis showed that enzyme treatment reduced the net negative surface charge trypsin about twice as much as neuraminidase. As regards neuraminidase, the release of sialic acid probably accounts for the decrease in mobility. It is surprising, however that the reduction in mobility was so small, considering the amount of sialic acid released. It is of the same order of magnitude as in other ascites tumours with greater reduction of the surface charge density (*Cook et al* 1962, *Miller et al.* 1963 *Weiss & Hauschka* 1970 *Codington et al.* 1973). One possible explanation would be that acidic groups regenerated after digestion. Yet, such regeneration should be minimal for cells stored at 0 C, especially since electrophoresis took place immediately upon digestion and washing (*Kraemer* 1966 *Collins et al* 1973). We find it more likely that deeply sited negative groups moved into the plane of shear (*Weiss* 1969 *Singer & Nicolson* 1972).

Trypsin may have released peptide-bound negative surface groups other than the small amounts of free sialic acid found by Warren's method. Hyaluronic acid for instance, may be such a constituent, since 101-AA cells produce a slimy material that recently has been demonstrated to be hyaluronidase-susceptible (unpublished observations).

We need to determine how much protein trypsin releases from the cells. Unfortunately these amounts are so small compared with the amount of protein in the enzyme itself that they elude detection by present methods (*Lowry et al.* 1951). At the present state of our knowledge, however we assume that trypsin changes the cell surfaces by removal of peptide-bound material, rather than by adherence to the surfaces. In fact the washing procedure used, especially in the presence of serum, should remove any adhering trypsin (*Weiss & Capes* 1966 *Poste* 1971).

When neuraminidase-treated cells were injected i.v., they gave rise to tumours distributed roughly as in control animals. In spite of a relatively great loss of neuraminic acid the cells thus retained their dissemination pattern. This finding matches the small in vitro changes detected after neuraminidase treatment.

Trypsin treatment caused a consistent change in the "metastasis" pattern induced i.v. The animals receiving trypsinized cells developed tumour masses in the lungs smaller than those in the controls. In Study A, performed with tumour generation 10, this was connected with a rise in the number of extrapulmonary tumours. In Studies B and C (gen. 28 and 39) there was also a greater number of extrapulmonary metastases (not statistically significant) but they were only found in muscle, preferentially in the myocardium. This rise in number of extrapulmonary metastases from trypsinized cells is due probably to a shunting of tumour cells past the lungs, thus explaining the smaller tumour mass in that organ. The finding is quite similar to results obtained by trypsinized suspensions of the solid ascites (AS) form of MCG101 (*Hagmar* 1974b) and explains why this tumour form behaved as an intermediate between the AA form (few extrapulmonary takes) and the SS form (many extrapulmonary takes). Similar effects of trypsinization have also been obtained when other tumours were used (*Boeryd* 1966, *Hagmar & Vesely* 1973). But to detect any such shunting of tumour cells through the lungs, it is essential to use a tumour that gives rise to extrapulmonary takes. Otherwise there will be only a reduction of pulmonary metastases (*Boeryd* 1966).

Boeryd (1966) suggested that reduced aggregability might be a mechanism by which

fewer cells would stick in the lungs. The trypsinized suspensions in the present experiments did in fact contain somewhat fewer aggregates than the other two types of suspension. Yet, in a study of another tumour (*Hagmar* & *Norrby* 1973) we found that aggregates increased the number of extrapulmonary metastases, but did not reduce lung tumour formation. Thus, while altered aggregability may be a factor we believe that the direct effects of trypsin on the cells is the more probable cause of the altered distribution of metastases.

If trypsinization facilitated the passage of tumour cells through the lungs, it obviously did not affect the affinity of the tumour to muscle as a site of metastasis. This property ("myotrophism") appeared also in the spontaneous spread of the solid ascites (AS) form of MCG101 (*Hagmar* 1974c). The myotrophism must be connected with the ascites conversion as it was not present in the original solid (SS) form of the tumour (*Hagmar* 1974a, b).

We assume that the tumour cells reach the muscles, including the heart, by capillaries where for some reason they are caught. Heart metastases, although growing intramurally, alternatively might arise from cells that adhere to the endocardium in the right side of the heart before passing the lungs. Exactly how they lodge in the heart and other muscles we still do not know. We are investigating this problem and other related problems by following the initial distribution of radio-labelled tumour cells and by testing other surface modifications of 101 AA cells. If, for instance, the peculiar "myotrophism" can be altered by surface modification, we will have a proof that surface features are essential for a certain metastasis pattern. It is our hypothesis that this may be true in a more general sense just as metastasizibility as such is probably cell surface dependent (*Coman* 1953, 1961). It is to be hoped that the clarification of specific surface factors connected with metastasis also will lead to therapeutic approaches.

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DIFFUSE LIPOMATOSIS IN THE LEG AFTER POLIOMYELITIS

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Kindblom, L.-G. & Möller-Nielsen, J. Diffuse lipomatosis in the leg after poliomyelitis. Acta path. microbiol. scand. Sect. A, 83 339–344 1975

Two elderly women with gradual enlargement of the leg following paralytic poliomyelitis are reported. From the clinical appearance, plain radiograms and angiographic studies the enlargement was thought to be caused by adipose tissue. On exploration the muscles within the calfs were found to be entirely replaced by adipose tissue. Histological examination revealed diffuse lipomatosis involving skeletal muscle, fasciae and in one case even periosteum and bone. Peripheral nerves exhibited fibrous and myelin degeneration and vessel walls showed several angioma-like changes. Since it is known from the literature that peripheral nerve lesions of different kinds can be followed by adipose overgrowth we believe that there is a pathogenetic relationship between poliomyelitis and the diffuse lipomatosis in the present cases.

Key words: Lipomatosis; poliomyelitis.

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According to the classification of soft tissue tumours elaborated by WHO (5) diffuse lipomatosis has been recognized as a subtype of benign tumours and tumour like lesions originating from adipose tissue. Diffuse lipomatosis occurs infrequently. In diffuse lipomatosis, the whole or part of a limb shows extensive infiltration of adipose tissue in the subcutaneous tissue, muscle, fascia and, in some cases, bone (6, 16). These changes are frequently associated with corresponding enlargement of bones (11, 16) and may coexist with osteochondroma (11), cavernous hemangioma (1) and multiple lipomas (6, 11, 12). The condition is usually encountered in children and is considered to be "exceedingly rare during adult life" (5).

Diffuse lipomatosis as well as multiple lipomas have sometimes been recognized as a manifestation of von Recklinghausen's neurofibromatosis (1, 8, 15). From experiments involving denervation, it is known that the skeletal muscle will be replaced by fat cells and that this process increases with time (2). A muscle paralysed by poliomyelitis frequently shows progressive fatty infiltration (4), a phenomenon known to be especially prominent in the gastrocnemius muscle (3). As far as we know, diffuse lipomatosis after poliomyelitis has not previously been described.

In the present paper two cases of diffuse lipomatosis are reported. Both patients were elderly women with progressive diffuse lipomatosis in the leg paralysed by poliomyelitis.

CASE REPORTS

Case 1. – A woman, aged 72 years, was hospitalized at the age of 19 years for poliomyelitis which resulted in paralysis of the right leg. Since then,

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rounding the tibia and fibula was possibly ised by cortical fragmentation and perio-l bone formation.

In the condition called "local gigantism" ich is characterised by enlargement of the nes of a whole or a part of a limb, adipose ue infiltration of the soft tissues is fre ently demonstrated (8 13) There is no ar distinction between diffuse lipomatosis th bone involvement and local gigantism. iffuse lipomatosis as well as local gigantism, pecially the variant with macrodactyly have en described as manifestations of von ecklinghausen s neurofibromatosis (1 8, i) "Bone softening" as seen in case 1 has en described both in neurofibromatosis (8, 5) and diffuse lipomatosis (16) In case 1 e softeness of the bone was demonstrated to e due to adipose overgrowth. In none of the resent cases there were signs of von Reck nghausen s neurofibromatosis or a family story of this disease.

Adipose overgrowth in extremities with amage to the nerve supply is known. Mul-ple subcutaneous lipomas in paretic extre-nities after spinal cord injury have been re-orted (7) and experimental muscle denerva ion may be followed by a progressive adipose nfiltration of the skeletal muscle (2) The erves in the two reported cases, especially ase 1 showed a pronounced epi- peri and endoneural fibrosis as well as myelin degener ation, i.e. changes which may appear in peri pheral nerves after poliomyelitis (4) Muscle tissue examined at different times after polio-myelitis may show an increasing adipose tissue infiltration (4) most pronounced in the leg (3) Diffuse lipomatosis after poliomyelitis, however is not known to us from the litera ture.

The angioma-like changes of vessels are believed to be secondary to the nerve lesions, as are the vessel changes seen in neurofibro-matosis (14) and not secondary to the adi-pose infiltration per se. It is of interest that a similar type of angioma-like wall structure of veins has been demonstrated in connection with cutaneous neurofibroma (9)

Thus, there are both experimental and



Fig 8 Case 1 Thick-walled vein showing elasti fibres extending into a fibrous tissue rich in capil lary-like vessels (Weigert's elastin-Haematoxylin van Gieson, × 120)

clinical observations indicating that peri pheral nerve lesions can be followed by adi pose overgrowth in extremities. We therefor believe that there is a pathogenetic relation ship between nerve lesions due to poliomye litis and the diffuse lipomatosis in the pre sent cases.

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EFFECT OF LONG TERM ADMINISTRATION OF DIFFERENT HARD LIQUORS AND RED WINE ON THE RAT LIVER

A Histological and Biochemical Study

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Jordö, L. & Olsson, R. Effect of long-term administration of different hard liquors and red wine on the rat liver. A histological and biochemical study. Acta path. microbiol. scand. Sect. A, 83 345–354 1975

Male rats were given 50 per cent of calories as ethanol, whisky, brandy, gin and red wine for 8–9 months together with moderate or low protein and vitamin supply. Histological studies at sacrifice failed to detect signs of hepatotoxicity but biochemical studies indicated that at least red wine and whisky produced more undesirable effects on the liver than ethanol.

Key words: Liver; rat; hard liquors; red wine; histology; biochemistry

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Earlier studies have failed to demonstrate any differences in acute hepatotoxicity between different alcoholic beverages (6).

Apart from the discussion whether alcohol or malnutrition is the cause of liver cirrhosis in the chronic alcoholic, it has long been disputed whether ethanol per se is the cirrhogenic factor or if other substances in different alcoholic beverages (congeners) may be of greater importance. Since no systematic study of liver morphology and biochemistry after long term administration of other alcoholic beverages than ethanol has been performed previously the present study was undertaken. Since malnutrition is common in the chronic alcoholic and possibly may increase the toxicity of congeners we contemporarily studied the effect of a low protein—low vitamin diet under these circumstances. In these experiments, rats were fed 50 per cent of total calories as ethanol, gin, brandy, whisky or red wine for 8–9 months in order to investigate if different alcoholic beverages differ in their effect on the liver.

MATERIAL AND METHODS

Male Sprague-Dawley rats, weighing 175–183 g at the start of the experiments were used for the study. Five animals were housed in every cage.

Diets. The rats were given semiliquid diets as the sole source of food and water. The diets contained 1.3 kcal/ml, and adequate water/calorie ratio for the rat (14).

Two different diets were used in which the calories derived from fat, protein, carbohydrate and ethanol were as follow (in per cent of total calories)

	Diet A	Diet B
Fat	6	20
Protein	14	6.5
Carbohydrate	30	23.5
Ethanol	50	50

As a basal diet we used a commercial rat diet (Astra-Ewos, Södertälje, Sweden) containing 28 per cent of calories as protein, 11.5 per cent as fat and 60.5 per cent as carbohydrates and the following vitamins (per kg diet):

Vitamin A	1,500 IU
D	1500 IU
E	50 mg
B_1	6 mg
B_2	14 mg
B_6	7 mg
B_{12}	0.07 mg
K_3	1.5 mg
Calcium pantothenate	15 mg
Niacin	70 mg
Choline chloride	1500 mg

The methionine content was 4.9 g/kg basal diet.

Diet A was prepared by mixing only the basal diet and alcohol, diet B by mixing cod liver oil glucose alcohol and the basal diet. The mixing was performed with a motor-driven mixer until an apparently homogeneous mixture was obtained. Mannucol SS 70 was used to stabilize the diet. The mixture was prepared once a week and kept in air-tight vessels which were shaken vigorously before they were opened. The food mixture was renewed every morning and left-over food mixture was regularly discarded. Since the main food consumption took place at day time, the weight-loss from the food mixture through evaporation of about 5 per cent during 24 hours was considered unimportant.

The lipotropic value of diet A was about 100 and that of diet B about 25 expressed as mg of choline/100 kcal according to *Port & Gomez Dumm* (16). The content of other vitamins were reduced correspondingly.

Alcohol was isocalorically given as ethanol, whisky brandy gin and red wine of *the pig station*. A control group received isocaloric amounts of glucose instead of alcohol. Each subgroup initially contained 10 animals.

Experimental procedures. During the experiments the food consumption was measured four days/week and the body weight was registered once a week. The serum ethanol concentration was determined in 15 animals from different subgroups after 6–8 months on the 50 per cent alcohol diets by aid of gas chromatography. The tail vein punctures were performed at different times of the day. A biopsy through laparotomy was performed in three animals from each group after 1 ½ and 3½ months. A renewed biopsy was performed in a small number of animals after 8 months. Since these did not show any marked differences compared to the 3½-month biopsies it was decided not to prolong the experiments further.

The animals were killed by exsanguination through aortic puncture under ether anaesthesia. During 24 hours before death the alcohol groups were reared on the diet of the corresponding control group. The rats in the present series were also subjected to other biochemical and morphological studies that will be presented elsewhere. Because of the time-consuming analyses which had to be performed immediately after sacrifice in all these animals the killing period had to be extended to one month. The killing of the animals in each subgroup was evenly distributed over this period.

Serum and liver obtained at biopsy or at sacrifice were kept at −20 °C until used for the biochemical analyses presented in this paper. A piece of the liver was fixed in 10 per cent neutralized formalin and stained with haematoxylin-eosin and with van Gieson's stain.

Analytical procedures. Triglycerides were determined according to *Carlson & Wadström* (4), serum cholesterol according to *Löfler* (11) and *Franey & Amador* (8). Liver cholesterol was determined essentially according to the method of *Zak et al.* (23), hydroxyproline according to *Bergman & Loxley* (3) in hydrolysates prepared according to *Woessner* (22).

Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined by *Ordell's* (15) modification of *Karmen's* (9) method using a commercial reagent, manufactured by AB Kabi, Stockholm, Sweden. One unit of enzyme activity is equivalent to a change in optical density of 0.001 per ml per minute at a wave length of 340 mμ. Serum ethanol was determined by aid of gas chromatography. Protein was determined according to *Weichselbaum* (21).

Paper electrophoresis of serum proteins was performed at pH 8.6 using a veronal buffer and bromphenol blue in ethanol solution. Liver lipid analyses were performed on liver tissue dried to constant weight in a vacuum pump. Liver hydroxyproline analysis was performed on dried tissue defatted through repeated washings in alcohol and ether.

All analyses were performed on duplicate samples except for serum electrophoresis and, because of shortage of material, serum aminotransferases.

Statistical methods. Non-parametric tests were used for the statistical comparisons. Wilcoxon's test for the ratios of liver and body weights and Fisher test for the other observations.

The five per cent significance level was used in

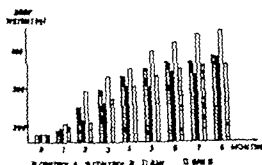


Fig 1 Pattern of body weight increase in controls A and B and gin A and B rats.

the following text, only differences significant on this level will be described. Absence of difference will be pointed out only when this is considered of particular interest.

RESULTS

Serum Ethanol

The serum ethanol concentration measured in 15 animals from different groups at different times of the day during the last two months varied between 55 and 270 mg/100 ml (mean 145 mg/100 ml). In spite of this, the animals did not appear intoxicated.

TABLE 1 *Mean Body Weight Increase/Month and M on Daily Consumption in Rats Given Different Alcoholic Beverage or Glucose (Controls) Corresponding to 50 per Cent of Total Calories.*

		Mean weight increase/month (g)	Mean daily food consumption (kcal/kg body weight)
Ethanol	A	35	241
	B	22	334
Brandy	A	27	248
	B	20	253
Whisky	A	28	244
	B	22	304
Gin	A	34	195
	B	21	288
Red wine	A	25	230
	B	25	280
Control	A	26	264
	B	27	285

Group A rats received a diet with a normal protein content whereas group B rats received a diet with low protein and vitamin content.

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Growth

Because of lack of spatial resources, pair-feeding was not possible, which would have been the ideal experimental approach. Therefore, the food consumption as well as the body weights were recorded for each cage of five animals, which makes statistical comparison of these parameters impossible. The mean monthly body weight increase during the whole experimental period (Table 1) seemed to be greater in the ethanol, gin, whisky and brandy rats given the A diet than the B diet. This difference might mostly be explained by the slow growth in the B animals during the first months of the experiments (Fig 1). For the sake of clearness Fig 1 only illustrates the pattern in controls and gin rats but the same tendency to difference between A and B groups as is evident in the gin rats was seen in all alcohol groups except red wine. Since differences in body weight increase could be caused by differences in food consumption it is also necessary to take this into consideration. A comparison of the daily food consumption and the body weight increase in the different groups (Table 1) shows that the greater body weight increase in the alcohol groups eating the A diet is not due to a higher caloric intake. Briefly the mean daily food consumption which was initially about 380 kcal/kg decreased with the growth of the animals to about 160 kcal/kg towards the end of the experiments. Thus, the mean daily alcohol consumption over the whole period was about 20 g/kg.

Mortality

Almost half the mortality occurred at the biopsies. When only the "spontaneous" mortality is considered it is apparent that there was a higher "spontaneous" mortality in the alcohol groups given diet B (28 per cent) than in the alcohol groups given diet A (8 per cent).

Liver Weight

The liver weight/body weight ratio was generally higher in the groups given the

	Diet A	Diet B
Fat	6	20
Protein	14	6.5
Carbohydrate	30	23.5
Ethanol	50	50

As a basal diet we used a commercial rat diet (Astra-Ewos, Södertälje Sweden) containing 28 per cent of calories as protein 11.5 per cent as fat and 60.5 per cent as carbohydrates and the following vitamins (per kg diet)

Vitamin A		12500 IU
"	D_3	1500 IU
"	E	50 mg
"	B	6 mg
"	B_2	14 mg
	B	7 mg
	B_{12}	0.02 mg
K_3		1.5 mg
Calcium pantothenate		15 mg
Niacin		70 mg
Choline chloride		1500 mg

The methionine content was 4 9 g/kg basal diet.

Diet A was prepared by mixing only the basal diet and alcohol, diet B by mixing cod liver oil, glucose, alcohol and the basal diet. The mixing was performed with a motor-driven mixer until an apparently homogeneous mixture was obtained. Manucol SS 70 was used to stabilize the diet. The mixture was prepared once a week and kept in air tight vessels which were shaken vigorously before they were opened. The food mixture was renewed every morning and left-over food-mixture was regularly discarded. Since the main food consumption took place at day-time, the weight-loss from the food mixture through evaporation of about 5 per cent during 24 hours was considered unimportant.

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Experimental procedures: During the experiments the food consumption was measured four days/week and the body weight was registered once a week. The serum ethanol concentration was determined in 15 animals from different subgroups after 6–8 months on the 50 per cent alcohol diets by aid of gas chromatography. The till vein punctures were performed at different times of the day. A biopsy through laparotomy was performed in three animals from each group after 1½ and 3½ months. A renewed biopsy was performed in a small number of animals after 8 months. Since these did not show any marked differences compared to the 3½-month biopsies it was decided not to prolong the experiments further.

The animals were killed by exsanguination through aortic puncture under ether anesthesia. During 24 hours before death the alcohol groups were reared on the diet of the corresponding control group. The rats in the present series are also subjected to other biochemical and morphological studies that will be presented elsewhere. Because of the time-consuming analyses which had to be performed immediately after sacrifice in all these animal the killing period had to be extended to one month. The killing of the animals in each subgroup was evenly distributed over this period.

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All analyses were performed on duplicate samples except for serum electrophoresis and, because of shortage of material, serum aminotransferases.

Statistical methods: Non-parametric tests were used for the statistical comparisons: Wilcoxon test for the ratios of liver and body weights and Fisher test for the other observations.

The five per cent significance level was used. In

TABLE 3 *Serum Analyse of Cholesterol, ASAT ALAT Albumin α-Globulin, β-Globulin and γ-Globulin at Sacrifice*

Group		Cholesterol (mg/100 ml)			ASAT (U/100 ml)			ALAT (U/100 ml)			Albumin (g/l)			α-globulin (g/l)			β-globulin (g/l)			γ-globulin (g/l)		
		n	x̄	S.E.M.	n	x̄	S.E.M.	n	x̄	S.E.M.	n	x̄	S.E.M.	n	x̄	S.E.M.	n	x̄	S.E.M.	n	x̄	S.E.M.
Ethanol	A	10	63.8	3.7	9	121	6	9	41	4	10	36	1	10	17	2	10	9.7	0.4	10	5.8	0.2
	B	5	45.2	3.8	5	122	12	5	43	4	5	30	2	5	18	2	5	9.9	1.5	5	5.7	0.4
Brandy	A	7	76.1	4.0	6	115	9	5	40	5	7	35	1	7	20	1	7	9.1	1.0	7	6.2	1.1
	B	9	65.3	3.2	9	124	6	9	48	5	9	33	2	9	19	1	9	10.4	1.2	9	4.7	0.4
Whisky	A	10	65.8	2.6	10	128	10	8	55	14	10	35	1	10	19	1	10	10.4	0.8	10	6.6	0.9
	B	5	59.6	5.8	5	161	9	5	58	7	6	32	1	6	21	2	6	12.4	1.6	6	7.1	0.9
Gin	A	6	69.8	5.3	6	92	12	6	51	5	7	37	2	7	17	1	7	8.5	0.7	7	6.0	0.8
	B	5	60.4	2.8	5	100	12	5	48	8	5	35	1	5	18	1	5	8.9	1.0	5	5.1	0.6
Red wine	A	7	79.9	3.2	4	149	12	4	84	31	7	36	2	7	18	1	7	10.9	0.8	7	6.5	1.0
	B	4	76.8	1.0	4	151	13	4	56	7	4	32	1	4	20	1	4	10.6	0.8	4	5.2	0.6
Control	A	8	*85.5*	5.9	6	*139*	10	6	38	5	8	35	1	8	20	1	8	9.1	0.5	8	5.1	0.3
	B	8	66.5	2.9	8	102	9	8	45	2	8	37	1	8	18	0.5	8	8.0	0.6	8	4.7	0.8

Italized value for A or B indicates a significantly higher value in comparison with the other (B or A)

B diet than in those given the A diet (Table 2) The gin rats given the A diet sometimes showed lower liver weight than the other A groups, whereas the gin rats given the B diet sometimes had higher liver weights than the other B groups.

Biochemical Analyses

Liver triglyceride analyses (Table 2) generally demonstrated higher values in the B group than in the A group. Although higher concentrations were often encountered in alcohol groups than in controls, no alcohol group showed a significant elevation at *all* times of the study Furthermore, the differences were never pronounced.

The *liver cholesterol* concentrations (Table 2) were usually higher in the B rats than in the A rats and higher in alcohol rats than in controls, but again, the differences were rather small.

Although alcohol groups usually showed higher mean values of *liver hydroxyproline* (Table 2) than controls, significant differences were only occasionally encountered.

A tendency to lower *serum cholesterol* values in B compared to A rats and in alcohol rats compared to control rats was sometimes statistically significant (Table 3) Among the alcohol groups, red wine generally had the highest mean values, and this difference was statistically significant in most of the comparisons. On the other hand, ethanol rats usually had lower values than the other groups.

Determinations of *serum ASAT* activity showed as a rule comparatively high levels in the red wine groups and low levels in the gin group (Table 3)

Serum ALAT determinations revealed no differences between the groups (Table 3)

Analyses of *serum proteins* (Table 3) showed only a trend to higher serum albumin levels in the alcohol A rats compared to alcohol B rats, whereas no such difference was discernible in the controls. The gin rats often had lower α and β-globulin levels than the other groups. Even though only a few statistical differences were detectable in the comparisons of the γ-globulin levels, the alcohol groups almost without exception showed higher mean values than the controls.

When summing up the differences observed in the comparisons of the different variables

TABLE 4 *Summary of Significant Differences in Tables 2 and 3*

Group				Control	Red wine	Gin	Whisky	Brandy	Ethanol
Ethanol	A	2	4			a f			
	B	2		c g					
Brandy	A	4	11	c		h	e		e
	B	7		b d f		c d			e
Whisky	A	6	16	c		a f h		c	c
	B	10		c f g h i		b f		f k	f
Gin	A	4	7	b c					b c
	B	3		a				a	e
Red wine	A	8	20	c		a f i	e	f	e f
	B	12		c d e f g h		d e f	e	f	e
Control	A	3	5			f h	e		
	B	2						e	e

In this summary an increased level is considered a pathological sign in all variables except serum albumin, where a low value was considered a sign of undesirable influence a) Liver weight, b) liver triglyceride at sacrifice c) liver cholesterol d) liver hydroxyproline, e) serum cholesterol f) serum ASAT g) serum albumin, h) serum α-globulin, i) serum β-globulin, k) serum γ-globulin.

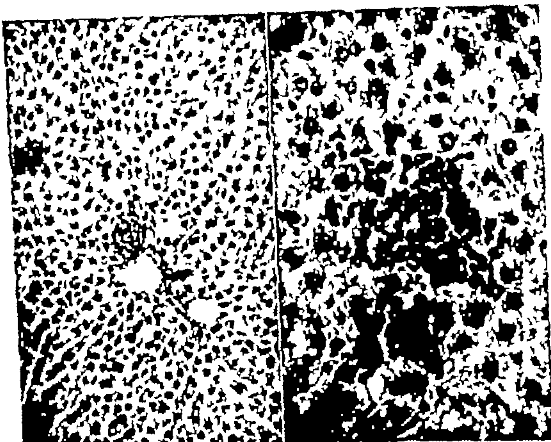


Fig. 2. Focal necrosis with lymphocytic infiltrates in otherwise normal liver parenchyma at sacrifice. a) control rat × 80, b) whisky rat × 200. Htx and eosin.

described in Tables 2 and 3 it is clear that red wine, whisky and brandy have shown more signs of undesirable influence than ethanol, which in fact did not seem to be more dangerous than large amounts of glucose (Table 4). Since the importance of an "undesirable influence" on one of the variables may not be comparable to that on another one, no attempt was made to statistically evaluate this summary. If we consider only the results of the determinations of serum ASAT and liver triglycerides (which may be considered to reflect "hepatotoxicity" better than the other variables used in the present study) we obtain the following figures for A and B: red wine 6, whisky 6, brandy 2, gin 2, ethanol 1 and glucose 1.

Liver Histology

Small isolated areas of necrotic liver cells with lymphocytic infiltration were seen in all groups without any differences in frequency or extent between the groups (Fig. 2). There was no preferable localization within the lobule of these necroses. No increase in fibrous tissue was apparent. A slight fatty infiltration was usually seen in the A groups receiving alcohol. This appeared somewhat more pronounced in the B groups. Since biochemical determination of liver lipids was performed no attempt was made to quantitate the histologically visible fatty infiltration.

DISCUSSION

As mentioned in the introduction, it has long been disputed whether ethanol per se is the

cirrhogenic factor or if other substances in different alcoholic beverages (congeners) may be of greater importance Since *Rubin* & *Lieber* have quite recently (19) demonstrated that alcoholic hepatitis and cirrhosis can be produced in baboons after very long periods of ethanol administration, it seems clear that congeners are at least not necessary for the evolution of liver cirrhosis in the chronic alcoholic. However there still remains the possibility that different alcoholic beverages, due to their congeners, may differ in hepatotoxicity For many reasons, it is very difficult to study the possible hepatotoxicity of congeners in man it is usually impossible to get a reliable history not only of the amounts of alcohol consumed but also of the type of alcoholic beverages possibly cirrhogenic differences between different alcoholic beverages may be obscured by simultaneous differences in duration of alcoholism, drinking habits, nutritional imbalancies, and alcohol amounts consumed. For example *Lelbach* (12) found that beer-drinkers usually consumed less alcohol per day than alcoholics drinking hard liquors.

It is evident that all the difficulties mentioned above can be overcome in animal experiments. Using experimental animals it is also possible to study simultaneously possible influences of variations in nutritional factors. Thus, it is our opinion that the experimental model used in the present investiga- permits a reliable comparison of the patotoxicity of different alcoholic beverages given to rats for long periods in very large amounts and under different dietary conditions. This comparison suggests that at least red wine, whisky and brandy may have more "undesirable" effects than ethanol. This is in accordance with observations by *Kiessling* & *Pihlström* (10) who studied liver mitochondrial respiration, oxidative phosphorylation, aerobic glycolysis and some enzyme activities in rats given various alcoholic beverages and observed some signs of more pronounced influence of wine and some hard liquors than of ethanol. These observations may indicate that congeners have adverse effects. However these were not sufficiently pronounced to produce a histologically detectable liver damage.

Some differences were detectable between the animals given an adequate diet and those given a diet deficient in protein and vitamins Thus, the rats given the low protein, low-vitamin diet seemed to have a slower growth but only when contemporarily given alcohol. Similarly alcohol provoked a higher mortality and a lower serum albumin concentration in the protein vitamin deficient diet. In contrast, the higher liver weight and the greater liver lipid accumulation occurring in controls B than in controls A was not constantly further accentuated by alcohol administration. This is in accordance with observations by *Porta et al* (17) that in rats given low or normal amounts of lipotropes, normal amounts of vitamins, 8 per cent of calories as protein and 13 per cent of calories as fat, ethanol does not cause more liver triglyceride accumulation than isocaloric amounts of sucrose. It thus seems reasonable to conclude that liver damage was not responsible for the high mortality encountered in the B group rats given alcohol.

Serum ASAT and liver triglycerides may be considered the best indicators of "hepatotoxicity" The sum of comparisons showing significantly higher values of these two variables induced by red wine, whisky brandy and gin in the A groups was 6 and in the B groups 10 Thus, it seems evident that the low protein, low vitamin diet did not markedly modify possible hepatotoxic effects of congeners. It is known that protein depletion considerably influences the effect of hepatotoxic agents. A compound such as CCl_4 which has to be metabolized by microsomal enzymes in the liver before rendered toxic is thus completely nontoxic in protein-starved animals (13) On the other hand, a substance which is rendered atoxic during metabolization in the liver can be expected to be more toxic if its metabolization is prevented by protein starvation.

The heavy fatty infiltration of the liver seen in chronic alcoholics was not observed

in the present study It seems likely that this lack of massive steatosis could be explained by the relatively low fat content of the present diets. In support of this is the higher liver fat content in the B rats compared to the A rats.

Although there was a tendency to higher liver hydroxyproline concentration in alcohol rats than in controls, the magnitude of these differences was rather small and only occasionally statistically significant. *Feinman & Lieber* (7) observed a 72 per cent increase of liver hydroxyproline concentration in rats after 7 months of ethanol feeding Although *Stromnachen* (20) has shown that the mesenchymal response to toxic liver injury increases with increasing levels of dietary protein, the difference in results between Feinman & Lieber and us is probably not explained by the higher dietary protein content in their experiments, for no clear difference between A and B rats in liver hydroxyproline concentration was detected in the present study

The low serum cholesterol in the B group animals is probably explained by the high content of polyunsaturated fatty acids in the cod liver oil given to the B group animals (18)

The minute histological changes found in all groups of animals are probably identical to those described by *Davis et al* (5) and by *Ahlqvist* (1) who suggest a parasitic origin.

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MYXOVIRUS-LIKE STRUCTURES IN THE GLOMERULAR ENDOTHELIAL CELL CYTOPLASM IN CANINE NEPHRITIS

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Krohn, K. & Sandholm, M. Myxovirus-like structures in the glomerular endothelial cell cytoplasm in canine nephritis. Acta path. microbiol. scand Sect. A 83 355–359 1975

Electron microscopic examinations revealed the presence of myxovirus-like tubular structures, 200A in diameter in the endothelial cytoplasm of the glomeruli in four dogs with chronic interstitial nephritis and in one healthy control dog. The structures were found inside the endoplasmic reticulum and in some longitudinal sections, the tubuli seemed to have a helical or spiral substructure. The morphology and cellular localization of the structures resembled those seen in systemic lupus erythematosus in man, and in cultures of cells from tissue infected with canine distemper. Precipitating canine distemper virus antibodies were not in evidence in dogs in which such structures were demonstrable by electron microscopy while these antibodies were found in other dogs.

Key words Canine interstitial nephritis myxoviruses.

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Tubular or spherical particles, morphologically resembling paramyxoviruses, have been found in human glomeruli in some pathological conditions, especially in connection with systemic lupus erythematosus (SLE) (1 2, 4 5 10) Similar particles have been found in some animal diseases, such as Aleutian mink disease (11) The character of these particles is unknown, but it has been suggested that they either might be viruses (1 4 5) or represent an unspecific cellular response to a variety of external stimuli (9 12).

We have studied the electron microscopic morphology of the glomeruli in chronic canine interstitial nephritis (CIN) In this disease, the glomerular lesions resemble those seen in SLE (6) In some of the electron micrographs, virus-like structures were observed which were subjected to a closer study

MATERIAL AND METHODS

Eighteen dogs were investigated. In six of these, aged from 6 to 13 years, a clinical diagnosis of chronic renal failure was established and chronic interstitial nephritis was confirmed by renal histology (6, 7) A control group was composed of four young dogs (aged ½–1 years) and of eight old dogs (aged 6–17 years) without clinical and histological signs of renal disease. All dogs had been vaccinated against canine distemper and canine infectious hepatitis (Candur SH, Boehring werke, Germany)

The dogs were anaesthetised intravenously with

thiopentone sodium (Intraval®) and samples were taken for electron microscopy and histology. Several pieces of about 1 mm³ were removed from the cortical region of each kidney and fixed for 60 minutes in 2.5 per cent glutaraldehyde pH 7.4 with 0.1 M phosphate buffer. After washing with the same buffer the samples were postfixed for 1 hour with 1 per cent osmium tetroxide and embedded in Epon 812. The sections were stained with uranyl acetate and lead citrate and studied using a Siemens A1 Elmiskope.

Precipitating antibodies in undiluted sera were assayed by micro-immunodiffusion in 1 per cent agarose in phosphate buffered saline, pH 7.2. A homogenate of culture of the Rockborn strain, distemper virus (kidney cell culture) was used as antigen. After 72 hours diffusion at room temperature, the plates were washed in PBS for 24 hours at +4 C and stained with amidoblack. By this method, about 90 per cent of all adult dogs admitted to the clinic for small animals at the College of Veterinary Medicine, Helsinki, were found to harbour precipitating antibodies.



Fig. 2. Electron micrograph of glomerular endothelial cell (EN) showing tubular structures inside the cisternae of the smooth endoplasmic reticulum (ER). The tubuli show a helical substructure. 100,000 ×



Fig. 1. Electron micrograph of glomerular endothelial cell showing aggregates of tubular structures in the cytoplasm. 28,000 ×

RESULTS

Paramyxovirus-like structures were seen in the endothelial cytoplasm of the glomeruli in one of the eight older control dogs and in four of the six nephritic dogs but in none of the four young controls.

Occasional longitudinal sections of tubuli of uniform size were seen, arranged in a fairly regular pattern (Fig. 1 and 3). The diameter of the tubuli was about 200Å and the distance between the tubuli in aggregates was about 100Å. Hexagonal formations were sometimes seen in which six tubuli surrounded one central tubular structure (Fig. 3). In longitudinal sections, helical form, resembling the nucleocapsid structures of paramyxoviruses, were observed (Fig. 2). The structures were located in the endothelial cytoplasm at the cisternae of the smooth endoplasmic reticulum.

Structures of similar appearance but at



Fig 3 Higher magnification of the area shown in Fig. 1 In some oblique sections, hexagonal arrangement of six tubuli around a single tubulus is seen. A smooth membrane continuous with the endoplasmic membrane lower most in the figure surrounds the aggregates 100 000 ×

ranged in strictly regular crystalline patterns, were observed in the mesangial cytoplasm of the two nephritic dogs (Fig. 4) These structures were also surrounded by the membranes of the endoplasmic reticulum.

The distribution of precipitating antibodies against canine distemper virus in the study group is shown in Table 1

DISCUSSION

Chronic canine interstitial nephritis is a disease which characteristically affects old animals of both sexes. A prominent histological alteration in the affected kidneys is the interstitial infiltration of lymphocytes and plasma cells. As a rule, however the glomeruli are also affected and the lesion in the glomeruli resembles the membranoproliferative nephritis seen in SLE (6) In previ

TABLE 1 *Occurrence of Precipitating Antibodies against Canine Distemper Virus in Nephritic and Control Dogs and the Presence of Myxovirus-like Structures in the Glomerular Endothelial Cells*

	Nephritic dogs (6–13 years)	Clinically healthy control dogs (½–1 years)	Clinically healthy control dogs (6–17 years)
Kidneys containing myxovirus-like structures	1/4	–	0/1
Kidneys without myxovirus-like structures	2/2	4/4	5/7

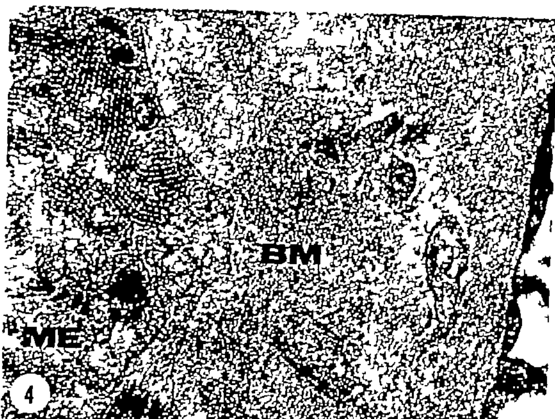


Fig. 4. Tubular structures arranged in a crystalline pattern in the cytoplasm of mesangial cell (ME) of the glomerulus. 60,000 × BM = basement membrane.

ous studies, the occurrence of electron dense deposits in the thickened glomerular basement membranes were seen, probably having a connection with the interstitial-glomerular nephritis in the dog (6). IgG type antibodies and the complement component C3 were deposited in the glomerular capillary wall (7). Elution and recombination studies showed that these antibodies probably originated from antigen-antibody complexes and that the antigen part of the complex was not endogenous and could thus be of viral origin.

The results of the present study indicate that there may be a connection between the renal disease and the presence of the paramyxovirus-like structures located in the glomerular endothelial cells. As regards the morphology these structures are similar to those occurring in SLE described earlier in various other collagen diseases in man and in some animal diseases, such as the Aleutian mink disease. It has been suggested that nuclear crystalloid structures in canine glomeruli may have some connection with infectious canine hepatitis (ICH). No doubt endothelial and mesangial cytoplasmic crystalloid structures can be found in apparently healthy dogs (3). Although it has been suggested that these structures could be due to various exogenous stimuli (9–12), it seems reasonable to assume that they represent viruses which could be of aetiological importance. Among the known canine viruses the most likely would be the distemper virus. In tissue culture this virus resembles morphologically the structures observed in the present study (8).

An inverse correlation between the finding of the virus-like structures in the endothelial cell and the occurrence of precipitating antibodies against distemper virus in the serum was observed.

It is possible that the immune response

against the virus normally occurring in vaccinated dogs fades in the course of ageing, thus leading to a continuous viral replication and excessive production of viral antigen. This process in turn would result in the formation of antigen-antibody complexes and might explain the presence of basement membrane-bound deposits in the glomeruli as observed in canine interstitial nephritis (7) and the disappearance of free (precipitating) antibody from the serum.

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INFLUENCE OF THYMECTOMY, TRANSFER OF THYMUS AND BONE MARROW CELLS AND TREATMENT WITH THYMOSIN ON THE DEPRESSED SPLENIC RELEASE OF LYMPHOCYTES INTO THE BLOOD AFTER IRRADIATION

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Ernström, U., Sandberg, G. & Björkholm, M. Influence of thymectomy, transfer of thymus and bone marrow cells and treatment with thymosin on the depressed splenic release of lymphocytes into the blood after irradiation. Acta path. microbiol. scand. Sect. A, 83: 360–368, 1975.

The content of lymphocytes in blood samples from the splenic vein and the splenic artery of guinea pigs was determined and the veno-arterial difference in number of cells was used as measure of the net release of cells from the spleen into the blood. The splenic release of lymphocytes was reduced after whole body irradiation with 300 rad. This reduction could partly be prevented by transfusion of bone marrow cells after irradiation, 10^9 cells being the most effective dose. In thymectomized, irradiated animals the restoration of the splenic release of lymphocytes after irradiation was impaired in comparison with sham-operated animals. In the thymectomized animals the transfusion of 10^8 thymus cells had a restitutive effect on the splenic release of lymphocytes, while transfusion of bone marrow cells or daily treatments with thymosin, alone or combined with transfusion of bone marrow cells, had no such restitutive effect. The results indicate that restoration of the splenic release of lymphocytes into the blood after irradiation is thymus dependent and probably caused by a traffic of lymphocytes from the thymus to the spleen.

Key words: Thymectomy; cell transfusion; thymosin; splenic release of lymphocytes; irradiation.

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In a series of papers the release of lymphocytes from the spleen into the blood has been quantitated in guinea pigs (*Ernström & Sandberg* 1968, *Sandberg* 1970, 1972). After a steroid-induced involution of the lymphatic tissue a complete restoration of the depressed splenic release of lymphocytes is dependent on an intact thymus (*Ernström & Sandberg* 1969). We have not tried to determine whether this is an effect of cells coming from the thymus or an effect of a thymic hormone.

In the present study an involution of the lymphatic tissue and a depression of the splenic release of lymphocytes are induced by whole-body irradiation. The restoration of the splenic release of lymphocytes after irradiation is compared in thymectomized and

sham-operated guinea pigs with or without protective infusions of bone marrow cells or thymus cells post irradiation. The possible restorative effect of a thymic extract, thymosin, is also explored.

MATERIAL AND METHODS

General experimental design. The splenic release of lymphocytes and granulocytes into the blood was estimated by the determination of the splenic veno-arterial difference in number of cells. Preliminary studies included firstly a radiation dose response experiment, secondly a time study of the effect of irradiation, and thirdly a comparative study of the protective effect of transfer of different number of bone marrow cells. In the main experiment the effect of transfer of bone marrow cells, thymic cells and treatment with thymosin was investigated in irradiated thymectomized and sham-operated animals.

Animals. Male non-inbred guinea pigs were used. The animals were fed on vitamin pellets, cabbage, turnips and carrots during the experiments and given water *ad libitum*.

Thymectomy and sham operation. Cutaneous infiltration anaesthesia with 0.5 per cent lidocaine (Xylocain® Astra, Sweden) was used. A small cervical incision was made in the skin and the two thymic lobes were resected. At sham operation the thymic lobes were mobilised but not resected.

Irradiation. Whole body irradiations were performed on groups of five animals in well ventilated plastic cages. The animals were gamma-irradiated from a ^{60}Co therapy unit at a distance of 90 cm. The dose rate was 100 rad per min.

Cell transfer. Guinea pigs weighing 300–500 g were used as cell donors. Bone marrow cells were taken from one femur suspended in saline and washed three times before adjustment of the cell concentration. The thymus of the donor animals was put into saline, cut into small pieces and passed through nylon nets for disintegration of cell aggregates. The thymus cells were washed three times and adjusted to 5×10^8 cells per ml. The reconstituting cells were given intracardially 15 h after irradiation in a volume of 0.2 ml.

Treatment with thymosin. The animals were given daily intraperitoneal injections with 0.5 mg of a partly purified calf thymus extract, thymosin fraction 3 (*Goldstein et al.* 1972) dissolved in 1 ml saline.

Preliminary studies. In a first experiment, different groups, each including five guinea pigs, were irradiated with doses from 150 to 600 rad and examined after 1 day. A distinct inhibition of the release of lymphocytes from the spleen was obtained with 300 rad and this dose was chosen for further experiments. In a time study groups of guinea pigs, each including 8 to 10 animals, were investigated from 1 to 15 days after 300 rad. In a third study the effect of different doses of reconstituting bone marrow cells, ranging from 10^3 to 10^8 cells per animal, were compared.

Experimental schedule. On the basis of the results obtained in the preliminary studies, the following schedule was used.

(1) *Cellular transfer to irradiated animals.* Guinea pigs weighing 210–230 g were irradiated with 300 rad, and given 10^8 thymus or bone marrow cells 15 h later. The animals were investigated 6 or 15 days after the cell transfusions, when weighing 260–310 g.

(2) *Cellular transfer to thymectomized irradiated animals.* Guinea pigs weighing 210–250 g were thymectomized or sham-operated, and 3 weeks later irradiated with 300 rad. Different groups of animals were given saline, 10^8 thymus cells or bone marrow cells 15 h after irradiation. The animals were investigated 6 days after the cell transfusions at a body weight of 360–480 g. It should be noted that all animals in this experiment were heavier at investigation than those in experiment 1 above with non-operated animals.

(3) *Thymosin treatment of thymectomized, irradiated animals.* Guinea pigs weighing 210–230 g were thymectomized or sham-operated and 3 weeks later irradiated with 300 rad. Different groups of 5 animals were then given daily injections of thymosin or saline and were investigated 6 days after irradiation. At sacrifice the weight of the animals was 380–460 g. No difference in body weight was found between sham-operated and thymectomized animals, treated with thymosin or saline.

(4) *Thymosin treatment of thymectomized irradiated and bone marrow reconstituted animals.* Guinea pigs weighing 220–240 g were thymectomized or sham-operated and 3 weeks later irradiated with 300 rad and 15 h later given 10^8 bone marrow cells. From the same day different groups of 10 animals were given daily injections of thymosin or saline. The animals were investigated 6 days after the reconstitution with bone marrow cells and they had then reached a weight of 350–480 g.

Investigational procedure. The following procedure was used for the determination of the spleno veno-arterial difference in number of lymphocytes and granulocytes. The animals were anaesthetized with 2.5 per cent pentobarbital sodium (25–50 mg/kg body weight, intraperitoneally). The spleen was exposed by opening of the peritoneal cavity between the last two ribs on the left side. 25 µl of blood was collected in a heparinized pipette (Heparin® Vitrum, Stockholm) after a small incision into the splenic vein, and immediately afterwards, 25 µl of blood from the splenic artery was with-

drawn by the same procedure. The blood samples were used for white cell counts in a hemocytometer after dilution with Tolsons solution (475 µl) Mono- and polynuclear cells were counted separately Blood was also collected in heparinized capillary tubes for determination of the hematocrit by centrifugation for 5 min at 9000 g

The number of nucleated cells in the left femur was determined in the following way Both ends of the left femur were cut and attached to a plastic tube. A syringe containing 1 ml saline was connected to one of the tubes. The content of the syringe was pressed through the central cavity of the femur and then sucked back again. This was repeated until all bone marrow cells were suspended. The suspension of cells was passed through nylon nets with decreasing mesh size in order to dissociate aggregates of cells. The total number of nucleated cells from the femur was calculated from the volume of the suspension and the cell concentration, determined by counting in a hemocytometer The wet weights of the spleen and the thymus were registered.

Statistical analysis. The difference in number of lymphocytes and granulocytes between blood from the splenic vein and splenic artery was calculated in the individual animal and the differences were analyzed by Student's t-test. All results are presented as the arithmetic mean and the standard error of the mean (mean ± S.E.)

RESULTS

Normal Animals

A group of normal, untreated guinea pigs was investigated in addition to the control groups of the separate experiments. A splenic veno-arterial difference of 979 ± 220 ($p < 0.001$) lymphocytes per µl was found in this group, thus confirming earlier results indicating a great release of lymphocytes from the spleen. A smaller splenic vendo-arterial difference of granulocytes was also found, 346 ± 148 ($p < 0.05$) cells per µl.

Dose Response Experiments

In a preliminary study different groups of guinea pigs were investigated 1 day after irradiation with 150 300 450 or 600 rad. As expected, the number of blood lymphocytes decreased with increasing dose of irradiation. The granulocytes, in contrast, were increased in number after the higher doses, 450 and 600 rad. The splenic veno-arterial difference in number of lymphocytes and granulocytes was markedly depressed after 300 rad (115 ± 54 lymphocytes per µl and 10 ± 98 granulocytes per µl) and higher doses. For the further experiments, a dose of 300 rad was chosen.

Time Experiments

In a second study guinea pigs were investigated at 1 3 6 9 12 and 15 days after irradiation with 300 rad. Both the blood

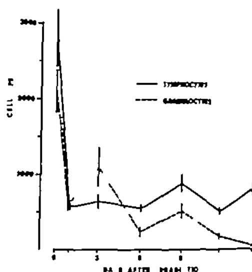


Fig 1 Content of lymphocytes and granulocytes in arterial blood at different intervals after irradiation with 300 rad. Mean ± S.E.

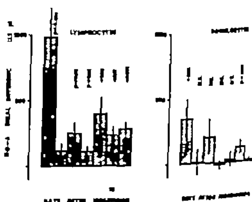


Fig 2 Splenic veno-arterial difference in number of lymphocytes and granulocytes at different intervals after irradiation with 300 rad. The p-values indicate the existence of a statistically significant release of cells from the spleen into the blood. Mean ± S.E.

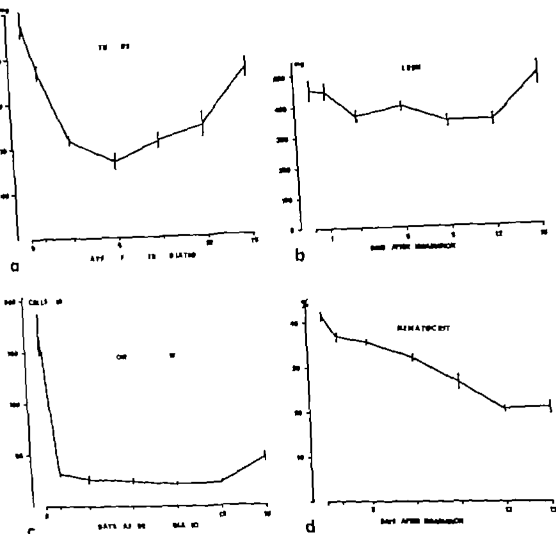


Fig. 3. Guinea pigs investigated at different intervals after irradiation with 500 rad. (a) Thymic weight. (b) Splenic weight. (c) Number of nucleated bone marrow cells per femur. (d) Hematocrit.

lymphocytes and granulocytes were below the normal level from day 1 and onwards. The blood granulocytes, in particular reached very low levels late after irradiation (Fig. 1). The splenic veno-arterial difference in number of lymphocytes and granulocytes was depressed (Fig. 2). The weight of the thymus was markedly decreased by the irradiation, the involution being maximal on day 6. Furthermore, the splenic weight, the cellularity of the bone marrow and the hematocrit were all depressed by the irradiation (Fig. 3). In the further experiments, the animals were investigated 6 and 15 days after irradiation.

Transfer of Different Doses of Bone Marrow Cells

Animals irradiated with 500 rad were given different doses of bone marrow cells. At investigation 6 days later the blood lymphocytes and granulocytes as well as the splenic veno-arterial difference in number of lymphocytes were found to vary with the number of bone

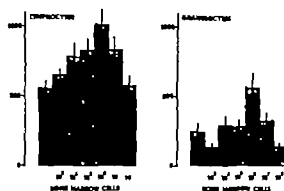


Fig 4 Effect of transfer of different doses of bone marrow cells on the content of lymphocytes and granulocytes in splenic arterial blood after irradiation with 300 rad. At investigation, 6 days after irradiation, the best restorative effect was obtained with 10^6 bone marrow cells. Mean ± S.E.

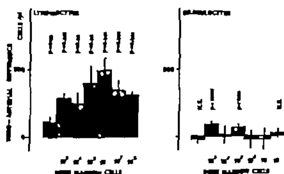


Fig 5 Effect of transfer of different doses of bone marrow cells on the splenic veno-arterial difference in number of lymphocytes and granulocytes after irradiation with 300 rad. The p-values indicate the existence of a statistically significant release of cells from the spleen into the blood. At investigation, 6 days after irradiation, the best restorative effect on the splenic release of lymphocytes was obtained with 10^6 bone marrow cells. Mean ± S.E.

marrow cells injected. Reconstitution with 10^6 bone marrow cells resulted in maximal number of blood lymphocytes and granulocytes and also the greatest veno-arterial difference (Fig 4 and 5). Though mutually different, a statistically significant splenic veno-arterial difference in number of lymphocytes per µl was found after all doses of bone marrow cells given. The number of transferred bone marrow cells did not influence the weight of the thymus and the spleen or the hematocrit. The cellularity of the bone marrow was lowest after reconstitution with the smallest number of bone marrow cells, whereas the other doses had equivalent effects in this respect. On the basis of the results, we decided to give 10^6 cells in the subsequent experiments.

Transfer of Thymus or Bone Marrow Cells

Animals irradiated with 300 rad were given 10^6 thymus cells or bone marrow cells and were investigated 6 or 15 days later. The animals were compared to controls not reconstituted with cells after the irradiation. The results are shown in Table 1.

The splenic veno-arterial difference in number of lymphocytes was depressed in the irradiated animals not given any cells. Significantly higher values were found after transfer of bone marrow cells. The effect of the bone marrow cells was most pronounced 6 days after irradiation ($p < 0.001$). Transfer of bone marrow cells also caused a higher level of circulating lymphocytes, and this effect was statistically significant at 6 days after irradiation ($p < 0.01$). Furthermore, bone marrow cells prevented the marked depression of the hematocrit ($p < 0.001$) and the decreased number of bone marrow cells ($p < 0.01$) found 15 days after irradiation. Transfer of thymus cells had no significant effect on any of these parameters.

Transfer of Thymus or Bone Marrow Cells to Thymectomized Animals

In this experiment, guinea pigs were thymectomized or sham-operated and 3 weeks later irradiated with 300 rad. Different groups of animals were given 10^6 bone marrow cells, 10^6 thymus cells or saline. The animals were investigated 6 days later. The results are summarized in Table 2.

Effect of thymectomy. Thymectomized, irradiated animals were compared with sham-operated, irradiated animals. The results showed a reduced cellular content in the spleen ($p < 0.05$), a reduced splenic veno-arterial difference in number of lymphocytes ($p < 0.05$) and reduced content of lymphocytes in the blood ($p < 0.01$) after thymectomy. The thymectomy did not influence the

TABLE 1 *Effect of Transfer of Thymic Cells (TC) or Bone Marrow Cells (BMC) to Irradiated Animals*

No. of animals	Transferred cells	Day of investigation	Splenic veno-arterial difference: Lymphocytes per µl	Splenic veno-arterial difference: Granulocytes per µl	Arterial blood: Lymphocytes per µl	Arterial blood: Granulocytes per µl	Arterial blood: Hematocrit	No. of cells per femur $\times 10^{-6}$
10	–	6	110± 39*	−20±33	553± 50	241± 65	32.1±0.8	25±3
10	10^8 TC	6	272±115	−75±32	758± 46	413± 59	34.8±0.7	23±1
9	10^8 BMC	6	493± 93	−18±71	1010±108	565±106	35.3±1.4	24±4
10	–	15	283± 50	11±17	814± 79	52± 12	21.1±1.3	47±4
9	10^8 TC	15	179± 86	−24±21	996± 58	118± 31	24.7±2.0	63±8
8	10^8 BMC	15	447±130	20±37	1079±114	93± 38	31.9±0.8	72±6

Guinea pigs were irradiated with 300 rad, given 10^8 TC or BMC, and examined 6 or 15 days later The best restorative effect was obtained with BMC as regards the splenic release of lymphocytes, the blood lymphocyte count, the bone marrow cellularity and the hematocrit.
* Mean ± S.E.

TABLE 2 *Effect of Transfer of Thymic Cells (TC) or Bone Marrow Cells (BMC) to Thymectomized or Sham-Operated Irradiated Animals*

	No. of animals	Transferred cells	Splenic veno-arterial difference: Lymphocytes per µl	Splenic veno-arterial difference: Granulocytes per µl	Arterial blood: Lymphocytes per µl	Arterial blood: Granulocytes per µl	Arterial blood: Hematocrit	No. of cells $\times 10^{-6}$ per spleen
Sham-operated	17	Saline	595±141*	168±52	962± 63	400± 56	37.5±0.5	102±9
Sham-operated	19	10^8 TC	320± 87	117±30	1122±163	608±126	35.3±0.8	90±8
Sham-operated	9	10^8 BMC	408±103	80±54	794±105	284± 40	36.4±0.9	N.D
Thymectomized	18	Saline	257± 61	158±58	635± 64	576±128	37.6±0.6	78±6
Thymectomized	19	10^8 TC	527± 96	108±43	716± 80	507± 75	36.7±0.6	102±9
Thymectomized	10	10^8 BMC	112±109	42±50	651± 59	230± 46	36.6±0.9	N.D

Guinea pigs were thymectomized or sham-operated and 3 weeks later irradiated with 300 rad, given 10^8 TC or BMC and examined after another 6 days. In the thymectomized animals a restorative effect on the splenic release of lymphocytes was obtained only after transfer of TC.
* Mean ± S.E.

cellularity of the bone marrow the hematocrit or the content of polynuclear cells in the blood after irradiation (Table 2)

Transfer of thymus cells Injection of thymus cells after the irradiation abolished the reducing effect of thymectomy on the splenic veno-arterial difference in content of lymphocytes becoming the same as in the sham-operated animals. Also the *reduced* content of cells in the spleen after thymectomy was compensated by giving thymus cells (Table 2) The amount of lymphocytes in the blood was not affected by this treatment.

Transfer of bone marrow cells. Transfer of bone marrow cells after the irradiation did not abolish the reducing effect of thymectomy on the splenic veno-arterial difference in content of lymphocytes, and did not influence the number of lymphocytes in the blood (Table 2) In the sham-operated animals, a less pronounced decrease of the splenic release of lymphocytes was caused by the irradiation than in the non-operated animals in the preceding experiment. The reason for this is the difference in age between the animals in these two groups and may explain

why transferred bone marrow cells had no demonstrable restorative effect in the sham-operated, irradiated animals.

Effect of Thymosin

In one experiment, guinea pigs were thymectomized or sham-operated and 3 weeks later irradiated with 300 rad. From one day after the irradiation, daily intraperitoneal injections of thymosin were administered for 6 days. The treatment with thymosin did not result in any elevation of the splenic veno-arterial difference in number of lymphocytes as compared to control animals injected with saline.

In a second experiment, thymectomized animals were treated as above, but given 10^8 bone marrow cells immediately before the first of the daily injections of thymosin. Nor in this experiment did the treatment with thymosin elevate the splenic veno-arterial difference above that in control animals given saline.

DISCUSSION

Studies on the effect of thymectomy in young immature animals have shown that the thymus is essential for proper development of the lymphoid system (*Miller* 1961 *Arnason et al.* 1962 *Martinez et al.* 1962) Thymectomy of more mature animals may also result in demonstrable deficiences provided that sufficient time is allowed to elapse before the animals are investigated (*Ernström* 1965 *Miller* 1965 *Taylor* 1965) If the lymphoid system is damaged by irradiation or steroids a deficient regeneration is usually seen in thymectomized animals (*Globerson et al.* 1962, *Miller* 1962) This fact has been utilized in the present paper for analysis of the importance of the thymus for the release of lymphocytes from the spleen into the blood.

Whole body irradiation with 300 rad caused a decreased release of lymphocytes from the spleen. When compared with earlier results this shows that the release of cells from the spleen is somewhat less radiosensitive than the release of lymphocytes from the thymus, which is markedly depressed by 150 rad (*Ernström* 1972) A similar difference in sensitivity to steroids has previously been demonstrated between splenic and thymic release of lymphocytes (*Ernström & Larsson* 1967 *Ernström & Sandberg* 1968). The irradiation with 300 rad had a more pronounced effect on younger animals than on older and heavier animals. Thus, irradiation depressed the hematocrit, the number of circulating lymphocytes and granulocytes and the splenic release of lymphocytes to a greater extent in younger animals (Table 1) than in older and heavier animals (Table 2)

Transfer of bone marrow cells after irradiation had a restoring effect on the splenic release of lymphocytes as well as on the number of circulating lymphocytes and granulocytes. The effect was dose dependent and optimal restitution was obtained with 10^8 bone marrow cells. Higher and lower doses were less effective. In contrast to the effect in animals with an intact thymus, bone marrow cells lacked a restorative ability in thymectomized animals. This fact seems to indicate that the effect was mediated by the thymus. There may have been stem cells among the transferred cells that permitted a faster repopulation of the thymus with subsequent cellular or humoral influence on the spleen. The reduction of the bone marrow cellularity and of the hematocrit late after irradiation was prevented by transfer of bone marrow cells, probably as a result of repopulation of the bone marrow with hematopoietic stem cells among the injected bone marrow cells. In this respect, thymic cells had no significant effect.

That the splenic release of lymphocytes was dependent on an intact thymus was further indicated by the more pronounced decrease of the release after irradiation of thymectomized animals than after irradiation of sham-operated animals. Furthermore, the transfer of thymic cells could compensate for the lack of the thymus. The reduced cellularity of the spleen found in the thymectomized animals was also prevented by thymic cells

Our experiments did not suggest any effect

of thymosin, neither alone nor in combination with bone marrow cells, on the restoration of the splenic cellular release after irradiation. The thymosin used was fraction 5 of *Goldstein et al.* (1972) which has been obtained from calf thymosin fraction 3 after a purification based on an assay in which the *in vitro* incubation of normal mouse bone marrow cells with thymosin results in cells with T-cell characteristics (*Bach et al.* 1971)

The cells transferred in our experiments were not syngeneic. This deserves a comment since it might be argued that the lacking genetic identity between donor cells and host cells might result in immune reactions interfering with the results. Firstly a graft-versus-host reaction (*Simonsen* 1962) might be caused by the transferred cells. However this is considered unlikely since bone marrow cells and thymus cells are reported to be relatively inert in this respect (*Yoshida & Osmond* 1971) Furthermore, the time interval between cell transfer and investigation is only 6 days and in fact no splenomegaly occurred even after 15 days. The possibility of an immune reaction of the host against the transferred cells can not be totally excluded although the animals were irradiated. However such a reaction is also less probable as the transfer of bone marrow cells had a restorative effect on the cellularity of the bone marrow and on the hematocrit, when examined 15 days after irradiation, suggesting that the transferred cells were not eliminated by a transplantation reaction at this time.

Our results indicate that the restoration of the release of lymphocytes from the spleen is dependent on an intact thymus and most probably due to a traffic of lymphocytes from the thymus to the spleen. Such a traffic has been demonstrated by labelling experiments in normal guinea pigs (*Murray & Woods* 1964 *Vossal & Gorris* 1964 *Linna & Stillström* 1966, *Ernström et al* 1971) A similar thymus dependency regarding the release of splenic lymphocytes was found during the regeneration after steroid-induced involution (*Ernström & Sandberg* 1969) The fact that bone marrow cells in our experiments are restorative only in the presence of the thymus suggests, that the transferred bone marrow cells pass through the thymus before they reach the spleen where they can influence the release of lymphocytes into the blood. The present work does not discriminate between two alternatives The transferred thymus cells, or the transferred bone marrow cells after passage through the thymus, may proliferate in the irradiated spleen, their descendants then being released. The other alternative is that the transferred cells influence the proliferation of remaining cells in the irradiated spleen, resulting in restoration of the release of lymphocytes from the spleen.

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RELATIONSHIP BETWEEN LENGTH OF THE LEFT MAIN CORONARY ARTERY AND HEART WEIGHT

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Paulsen, Stein, Vetner, Max & Hagerup, Leif M. Relationship between length of the left main coronary artery and heart weight. Acta path. microbiol. scand. Sect. A, 83: 369–372, 1975.

The length of the left main coronary artery (LMA) was determined in post-mortem, non-fixed hearts of normal weight (heart weight < 400 g, group I) and hypertrophic hearts (heart weight $\geqq 400$ g, group II). Hearts from 76 men and 88 women in group I and 156 men and 61 women in group II were studied.

The mean length of LMA in group I was 9.1 mm in men and 8.4 mm in women. Corresponding values in group II were 10.3 mm and 8.8 mm. There was no significant sex difference in the length of LMA in group I, but the sex difference in group II was significant ($p < 0.01$). The difference in length of LMA between groups I and II reveals that the difference in men was significant ($p < 0.02$), but insignificant in women.

In the case of both sexes no significant correlation between heart weight and the length of LMA was found either in group I or group II. There was a significant correlation between the area of the left coronary ostium and the length of LMA in men in group I ($p<0.05$), but not in women. Corresponding comparisons of both sexes in group II were statistically insignificant.

Key words: Coronary artery length; heart weight.

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The left main coronary artery (LMA) represents the portion of the left coronary artery running from the left coronary ostium to the point where the artery divides into the circumflex branch and the left anterior descending artery. The length of the LMA plays an important role in cardiac surgery since cannulation of the left coronary artery is employed in artificial perfusion of the coronary circulation. Few studies have dealt with the numerical size of the LMA. Search of the literature has uncovered only two papers on this problem (*Green et al.* 1967, *Fox et al.* 1973). The purpose of the present study was 1) to determine the length of the LMA in a group of hearts of normal weight and a group of hypertrophic hearts, 2) to determine whether there is a correlation between heart weight and the length of the LMA, and 3) whether the area of the left coronary ostium is related to the length of the LMA.

MATERIAL AND METHODS

Four hundred unfixed hearts consecutively collected at autopsy over a 9 month period were studied. These hearts were divided into two groups, group I where heart weight was < 400 g and group II with heart weight $\geqq 400$ g. Thirty-nine hearts were

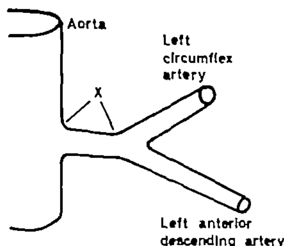


Fig 1 Diagram illustrating the measurements of the left main coronary artery at necropsy

discarded, two because of anatomical anomalies of the coronary circulation and the remaining 37 because of technical difficulties in measuring the area of either the left coronary ostium or the length of the LMA, primarily because of atherosclerosis. Measurements were performed by a pointed compass by the same investigator (M. V.) The portion of the artery measured, LMA, is shown in Fig. 1 Measurement of the left coronary ostium consisted of estimation of circumference and thereafter calculation of the area.

Statistical calculations were performed by means of Kruskal Wallis and Spearman's rank correlation test. The level of statistical significance chosen was $p \leq 0.05$

RESULTS

Age and sex distribution in the two groups is given in Table 1

Group I

The values applying to mean age, heart weight, area of left coronary ostium and length of LMA are given in Table 2. Distribution of length of LMA in men and women is presented graphically in Fig. 2.

The length of LMA showed no significant sex difference and correspondingly the difference in the length of LMA in the various age groups in men and women was statistically insignificant.

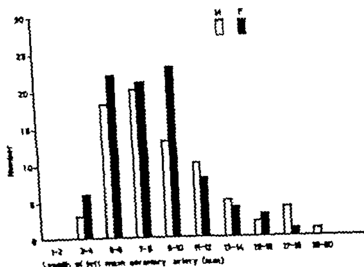
TABLE 1 *Age and Sex distribution of the Studied Series*

Age group	Men Heart weight		Total	Women Heart weight		Total
	< 400 g	≥ 400 g		< 400 g	≥ 400 g	
< 40	7	1	8	4	0	4
40–49	10	3	13	9	2	11
50–59	4	22	26	16	2	18
60–69	27	47	74	22	20	42
70–79	21	40	61	25	29	54
> 80	7	23	30	12	8	20
Total	76	136	212	88	61	149

TABLE 2. *Sex Distribution, Average Age, Mean Heart Weight, Area of the Left Coronary Ostium and the Length of the Left Main Coronary Artery in Group I (Heart Weight < 400 g)*

Sex	Age (years) mean	No. of hearts	Heart weight (g) mean ± SD	Left coron. ost. (sq mm) mean ± SD	Length of left main coron. artery (mm) mean ± SD
M	62	76	318 ± 47	7.7 ± 2.9	9.1 ± 3.6
F	65	88	296 ± 65	7.8 ± 2.9	8.4 ± 3.1
Total	62	164	306 ± 58	7.7 ± 2.9	8.7 ± 3.4

Fig. 2. Length of the left main coronary artery in group I (heart weight < 400 g)



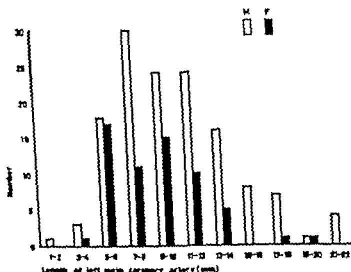


Fig 3 Length of the left main coronary artery in group II (heart weight ≧ 400 g)

In both sexes, no relationship was found between heart weight and length of LMA. Relationship between area of the left coronary ostium and length of LMA in men and women revealed correlation in men ($p < 0.05$) but not in women.

Group II

The values applying to average age heart weight, area of the left coronary ostium and length of LMA are given in Table 3 Distribution of length of LMA in men and women is graphically presented in Fig. 3.

As can be seen in Table 3 and Fig 3 hypertrophy of the myocardium is associated with an increase in length of the LMA in men, whereas increase in length in women is very small. The difference in the length of LMA in men and women is statistically significant ($p < 0.01$) Corresponding to the findings in group I age had no influence on the length of LMA.

The difference in the length of LMA in subjects in group I and group II is statistically significant in men ($p < 0.02$) but not in women.

TABLE 3 Sex Distribution, Average Age, Mean Heart Weight, Area of the Left Coronary Ostium and the Length of the Left Main Coronary Artery in Group II (Heart Weight ≧ 400 g)

Sex	Age (years) mean	No. of hearts	Heart weight (g) mean ± SD	Left coron. ost. (mm²) mean ± SD	Length of left main coron. artery (mm) mean ± SD
M	66	136	542 ± 103	9.9 ± 4.6	10.3 ± 4.0
F	72	61	463 ± 97	9.0 ± 3.8	8.8 ± 3.2
Total	69	197	524 ± 103	9.6 ± 4.4	9.9 ± 3.8

No significant correlation between heart weight and the length of LMA was found in either men or women, but in the series as a whole, correlation was found to be significant at the 5 per cent level. In both sexes there was no significant relationship between area of the left coronary ostium and the length of LMA.

DISCUSSION

Green et al. (1967) measured the length of LMA in 50 consecutive unfixed hearts in an autopsy study. Their series was not classified according to sex and hearts of normal weight as well as hypertrophic hearts (range 240–550 g) were included. The mean length of LMA in this study was 11.6 mm. In 52 per cent of the cases, the length of LMA was greater than 10 mm and in 26 per cent of the cases it was ≦ 6 mm. Corresponding figures in our study were LMA > 10 mm in 31.7 per cent of the cases (95 per cent confidence limits 26.5–37.7 per cent) and in 24.7 per cent ≦ 6 mm (95 per cent confidence limits 20.3–27.9 per cent). *Green et al.* found no correlation between the length of LMA and the diameter of the coronary ostia or between the length of LMA and heart weight. *Fox et al.* (1973) measured the length of LMA in 100 consecutive coronary angiograms. They found a mean length of LMA of 9.5 mm. In 36 per cent of the cases, the length was ≦ 6 mm, being > 20 mm in 5 per cent of the cases. In addition, the length of LMA was measured in 100 formalin fixed post mortem hearts of normal weight. In the autopsy study a mean length of LMA of 5.5 mm was found and in 10 per cent of the cases the length was 0 mm being ≦ 6 mm in 73 per cent in 5 per cent of the cases the length was > 10 mm. Corresponding figures obtained in our study of patients in group I showed that a heart where the length of LMA was 0 mm was never observed, whereas in 29.9 per cent (95 per cent confidence limits 23.9–37.5 per cent) the length was ≦ 6 mm and in 23.2 per cent (95 per cent confidence limits 16.9–30.3 per cent) > 10 mm. Comparison of the results obtained by Fox et al. with those obtained in the present study demonstrates some agreement between findings in our study and the angiographic results on the other hand, there was a marked difference between the post-mortem findings and our values in group I. Some of the difference may have been due to the formalin fixation of hearts which was carried out in Fox's et al.'s study. Separation on the basis of sex had not been undertaken in any of the two studies referred to above.

Statistical calculations were carried out at the Department of Medical Data Processing, Gentofte Hospital, 2820 Gentofte DK.

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ON THOROTRAST LEUCAEMIA

Evolution of Clone of Bone Marrow Cells with Radiation Induced Chromosome Aberrations

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Visfeldt, J Jensen, G & Hippe, E. On Thorotrast leucaemia. Evolution of clone of bone marrow cells with radiation-induced chromosome aberrations. Acta path. microbiol. scand. Sect. A, 83 373–378, 1975

Results are presented of chromosome studies of bone marrow cells from a 62-year-old woman. The patient had been given 40 ml of Thorotrast in connection with a neuro-radiological examination 34 years earlier. Clinically the patient was now considered to be in an incipient myeloid leucaemic phase. Ninety-seven per cent of the bone marrow cells belonged to a clone with characteristic marker chromosomes induced by radiation. Few data are available on chromosome analysis of bone marrow cells from Thorotrast patients. Therefore, the results of the present study are compared with data from chromosome analyses of ^{32}P treated patients with polycythaemia vera, in whom large clones were found in the bone marrow. The results of chromosome analysis of bone marrow cells from the Thorotrast patient support previous hypotheses concerning the carcinogenesis in radiation-induced leucaemia in patients with polycythaemia vera, treated with ^{32}P. In the latter patients, the clone cells are presumed to represent cell populations with selective qualities, originating from radiation-damaged cells, which possibly possess an increased tendency to malignant transformation because of a more pronounced sensitivity to carcinogenic agents. The cells might also be potentially malignant and manifest themselves as leucaemic cells, if the patient immunological defence mechanism is broken.

Key words Thorotrast chromosome aberrations leucaemia.

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The development of leucaemia following application of Thorotrast is well known from reports available on comprehensive Thorotrast series. The Danish series comprises 1005 Thorotrast patients, 756 of whom survived the primary disease. Out of the 756 patients, 312 have died. Eleven of these had leucaemia (5)

So far few results have been published on chromosome analyses on bone marrow from Thorotrast patients.

We have had the opportunity of assessing the chromosome aberrations in a Thorotrast patient who, from a clinical point of view was considered to be in an incipient leucaemic phase. The results of this study are presented, as they may contribute to a clarification of the carcinogenesis in radiation-induced leucaemia.

CASE REPORT

The patient, a 62-year old female was referred to hospital in January 1974 suspected of suffering from leucaemia.

Thirty-four years earlier she had undergone a neurological examination because of diplopia, headache, and epileptic absences. Radio-arteriography was performed on three occasions within one month, in order to exclude a cerebral tumor. A total dose of 40 ml of Thorotrast was injected.* The diagnoses were sequelae of arachnoiditis, symptomatic epilepsy and paresis of the left lateral rectus muscle of the eye. Subsequently the patient was treated with 300 mg of phenytoin daily.

The symptoms and signs of the present disease were increasing fatigue, sweating, and a weight loss of 10 kg over a period of six months. Laboratory data obtained by her family doctor revealed a haemoglobin of 5.4 mmol/l and a white cell count of 6.2 × 10^9/l. A peripheral blood smear showed about 10 per cent immature myeloid cells.

On physical examination in hospital, the patient appeared tired, and the state of nutrition was below normal (body weight 47 kg, height 148 cm). The skin and the mucous membranes were pale, but without jaundice or haemorrhages. No palpable lymph nodes were found, and neither the liver nor the spleen were enlarged.

The patient was observed for three months until her death, and during this period the condition of her disease was apparently stable.

The anaemia could not be explained by simple loss of blood. It seemed rather to be of a haemolytic nature. The haemoglobin varied from 5.0 to 5.6 mmol/l, the erythrocyte count from 2.7 to 3.1 × 10^{12}/l, MCV from 87 to 99 fl and MCHC from 18.3 to 19.2 mmol/l. The reticulocyte count was increased, i.e. from 42 to 310 × 10^9/l, and a few erythroblasts were seen in the blood smears. The erythrocytes presented increased osmotic fragility and an increased content of some intracorpuscular enzymes (i.e., glucose 6-phosphate dehydrogenase, pyruvate kinase and ATPases). Coombs' antiglobulin test, as well as the Ham, Crosby and Donath-Landsteiner haemolytic tests were all negative. The thrombocyte counts ranged between 30 and 92 × 10^9/l, and the leucocyte counts between 4.0 and 24.2 × 10^9/l. The serum cobalamin was normal (346 pmol/l) as well as the serum lysozyme (260 nmol/l).

The bone marrow was hyperplastic and contained large quantities of Thorotrast deposits. No fibrosis was observed, and the iron content was very low. Smears of bone marrow were rich in cells, with 24 per cent erythroblasts and normoblasts. Promyelocytes, myelocytes, and metamyelocytes made up 31 per cent in all. Eight per cent were classified as myeloblasts. From the same bone marrow aspirate, material was used for chromosome analysis, prepared according to a modification of the direct method of *Tjio & Whang* (11).

The smear of peripheral blood, prepared on the same day as the bone marrow sample, presented 49 per cent granulocytes, 30 per cent lymphocytes, and 3 per cent monocytes by differential counting. Ten per cent were *erythroblasts* and normoblasts. Myelocytes and metamyelocytes amounted to 3 per cent. Five per cent were classified as myeloblasts. At the same time, peripheral blood was withdrawn for chromosome analysis, the short-term method of *Moorhead et al.* (9) being used.

It was concluded that the haematological disease most likely was induced by the Thorotrast irradiation and that the condition was preleucaemic. However we found no indication for cytostatic treatment at present, but tried to influence the haemolytic state by administering prednisone (20 to 30 mg per day for 2½ months) without success. Further examinations were planned (erythrocyte and thrombocyte survival studies, repeated chromosome analyses on bone marrow including banding technique) but the patient died suddenly of a stroke in her home. Autopsy was not performed.

RESULTS

Chromosome Patterns of the Bone Marrow Cells

One hundred cells of good quality were analyzed. Only two cells presented normal karyotype. One cell had 44 chromosomes and could not be classified accurately.

Of the remaining 97 cells, the aberrations fell into two main groups with the following karyotypes:

I 46,XX, inv (1?–?+) –B,–B,+C,+C, –D,F?–?– +G,dic.
The dicentric chromosome was counted as two chromosomes when recording the number of chromosomes.

II 44,XX, inv (1?–?+) –B,–B, D,F?–? +G

Morphologically the dicentric chromosomes were identical in the cells concerned. The chromosome No. 1 with the pericentric inversion was also morphologically identical in the various cells. Both aberrations are characteristically radiation-induced.

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* Verified by the Danish Thorotrast Register. Professor *M. Faber*. The Finsen Laboratory. Copenhagen.

Some of the cells presented have various other abnormalities as well. There were different chromosome numbers and aberrations, which were supposedly induced by radiation, but these varied from one mitosis to another.

In 5 cells, there were acentric fragments of varying size. Four of these cells were among those with a dicentric chromosome, although there was no evidence of any relation.

Table 1 shows the distribution of the cells, first as regards the dicentric chromosome and the inversion (1?–?+) and secondly as regards the number of chromosomes.

The plate showing Figs. 1–2 and 3–4 presents four survey pictures of mitoses. The two sets of pictures show the two largest groups of Table 1 clone cells with and without a dicentric chromosome, respectively.

TABLE 1. *Chromosome Patterns of 100 Bone Marrow Cells 97 of Which Present a Clone*

No. of cells	No. of chromosomes	Pericentric inversion	Dicentric
2	46		
1	44		
27	46	1	1
1	46		1
6	45	1	1
2	44	1	1
1	44	1	2
1	43	1	1
6	47	1	1
1	48	1	1
1	48	1	2
1	52	1	1
1	53	1	2
23	44	1	
3	43	1	
17	45	1	
3	46	1	
1	47	1	
1	49	1	
1	51	2	

Chromosome Patterns of Cells from Peripheral Blood

One hundred cells of good quality were analyzed. Eight aneuploid cells, and 18 cells with various stable and unstable structural chromosome aberrations were found, these aberrations being considered to be radiation-induced. Only one of the 18 cells originated from the clone found in the bone marrow with 46 chromosomes, one dicentric chromosome, and one inversion (1?–?+).

DISCUSSION

Chromosome Analyses from Thorotrast Patients

Chromosome changes in Thorotrast patients have been reported in few publications. In almost all cases the chromosome analyses were carried out on peripheral blood after short term cultivation, in which cases the lymphocytes are found to be in mitosis. The most important object was generally to investigate a possible relationship between chromosome aberration frequency and Thorotrast dose. However the results obtained varied in the different investigations.

The report published by *Buckton et al.* on the finding of clones in peripheral blood (lymphocytes) in three Thorotrast patients (2, 3) is of particular interest, even though these clones included only a small percentage of the cells.

In Thorotrast patients, chromosome analyses of bone marrow have been performed only on rare occasions, and no comprehensive series have been published so far. *Trübestein & Citoler* (12) reported a case of chronic myeloid leucaemia, where 20 bone marow cells were analyzed and presented the Philadelphia chromosome, but no other chromosomal abnormalities. When studying four Thorotrast patients, *Hennekeuser et al.* (4 8) found no chromosome aberrations in the bone marrow cells.

Chromosome analyses of the bone marrow from our patient show that 97 per cent of the cells belonged to one clone. Hence, the clone cells possess pronounced selective qualities. Generally dicentric chromosomes will appear only after irradiation and will be associated with an acentric fragment. They are most often "unstable" i.e., they will be lost or will complicate the division of the cell to such an



Figs. 1–4 Bone marrow cells in metaphase.

Figs. 1 and 2 Clone cells with 46 chromosomes, one chromosome No. 1 with pericentric inversion, and one dicentric chromosome.

Figs. 3 and 4 Clone cells with 44 chromosomes and one chromosome No. 1 with pericentric inversion, one dicentric chromosome.

The chromosome No. 1 with pericentric inversion and the dicentric chromosome are indicated by arrows.

extent that the cell will not survive the first division. However, in a few cases the cell can pass through one or more mitoses, still having a dicentric chromosome. An acentric fragment will soon be lost during cell divisions. Half the clone cells have lost the dicentric chromosome (see Table 1). As a result, important genetic material is lost, and for benign cells this will normally not be compatible with continued viability.

Chromosome Studies of ³²P-treated Patients with Polycythaemia Vera

As no sufficiently comprehensive studies are available of chromosome aberrations in bone marrow cells from Thorotrast patients, we compared the results of the present study with data from bone marrow studies of ³²P treated patients with polycythaemia vera (13, 14, 15). In a great many ³²P treated patients, large clones of bone marrow cells are found with abnormal chromosome patterns. It is presumed that the clones originate from cells with radiation-induced chromosome abnormalities. The clone cells must possess selective qualities, because the clones are often very large. The clone has been seen to increase to 100 per cent when the condition approaches the terminal stage. Generally, the clinical picture is that of acute or subacute myeloid leucaemia at the terminal state.

The hypothesis has been advanced, as regards the ³²P treated patients, that the clone cells represent populations which might have an increased tendency to malignant transformation so that a connection may exist between the chromosomally abnormal bone marrow cells and the increased incidence of leucaemia in patients with polycythaemia vera.

Carcinogenesis in Thorotrast Induced Leucaemia

The time lag is variable between the administration of Thorotrast and the development of leucaemia. *Grebe* (7) describes a case of leucaemia with a latent period of only 6 years. *Trubestein & Citoler* (12) reports a case of leucaemia 36 years after Thorotrast injection. It seems that both myeloid and lymphatic types of leucaemia may develop. Erythro-leucaemia has also been described (1). The case presented in this paper is considered, from a clinical point of view, to be an incipient transition into myeloid leucaemia. Both in the bone marrow and in the peripheral blood a limited number of blast cells of myeloid type were found. In this connection it should be mentioned that during development of leucaemia in ³²P treated patients with polycythaemia vera, the incipient transition into leucaemia manifests itself in a similar way, often by the appearance of a few blasts in the bone marrow and in the peripheral blood. These single blast cells might for years be the only sign of an early leucaemic phase (6).

Although, in the present case, there are no definite reasons for suspecting malignant transformation of the clone cells. The results seem to indicate the same pathogenesis for the radiation-induced leucaemia following Thorotrast injection, as that advanced hypothetically for the ³²P-induced leucaemia in the polycythaemia vera patients (13). In the latter case, the clone cells are presumed to represent cell populations with selective qualities, originating from radiation-damaged cells, which possibly have an increased tendency to malignant transformation because of a more pronounced sensitivity to carcinogenic agents. The cells might also be potentially malignant and manifest themselves as leucaemic cells, if the patient's immunological defence mechanism is broken (10).

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SEMIMACROSCOPIC EXAMINATIONS OF THE SURFACE PATTERN OF SMALL INTESTINAL MUCOSA IN CROHN'S DISEASE

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Poulsen, S. S., Nielsen, O. V. & Fabricius-Lauritzen, K. E. Semimacroscopic examinations of the surface pattern of small intestinal mucosa in Crohn's disease. Acta path. microbiol. scand. Sect. A, 83: 379–388, 1975.

The mucosal surface pattern of surgical specimens from small intestine affected by Crohn's disease are studied using Alcian-green staining of whole mounts. The semimacroscopic appearance of the mucosa is described. Our findings include i.a. malformation and enlargement of villi—often to extreme degree—villi frequently showing small vesicles on their apical region. Polypoid structures were found to be svillous as well as covered by normal or abnormal villi. The semimacroscopic observations are compared with observations from conventional light microscopy. The villus enlargement is in the first stages caused by lymphatic dilatation and later by oedema and inflammation.

Key words: Intestinal mucosa; small intestine; surface pattern; Crohn's disease.

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In 1932 *Crohn et al.* (2) described regional enteritis as a clinical entity. Since then, the histopathological changes characteristic of this disease have been described in a great number of papers (1, 5, 6, 7, 8, 9, 12, 13). A variety of changes has been observed, but they are seldom all seen in specimens from a single patient. No changes are pathognomonic for the disease (1, 5) and in several cases the histological diagnosis is a tentative one.

It has been the aim of the present study to add to the number of previous studies which mainly are based upon findings in conventional histological sections, by using the method of direct study of the intestinal surface after staining of whole mounts (4). The findings were subsequently supplemented by studies of the specimens, using conventional histological techniques.

MATERIAL AND METHODS

The series comprises six patients with Crohn's disease in the age group 16–30 years.

The predominant symptoms were pain, diarrhoea, and loss of weight. In four patients, a palpable intra-abdominal mass was present. Internal fistula or fistula in ano were found in four patients.

Half of the patients had been submitted to two or more operations. Large segments of small bowel has been resected in the majority of patients, in some 200 cm.

The histological diagnosis of Crohn's disease was in all cases established by the pathologists by conventional light microscopy. In all cases transmural inflammation with lymphocytes as the pre-

25*

dominant cells was present. Ulcerations and thickening of the bowel wall (often measuring more than cm) were present too. Granuloma, cobblestone pattern, and fissures were described in most cases.

From two of the patients, two specimens were removed and from the remaining four patients, one specimen.

Technique of preparation for the semimacroscopic surface examination. Specimens from the diseased small bowel (approximately * × 5 cm) were removed during the operation, before the vessels to this segment were clamped. The specimen was immediately immersed in cold buffered 4 per cent glutaraldehyde (formaldehyde may be used too) and after short fixation suspended with fine needles on a polyethylene plate. After 48–72 hours fixation, the specimen was transferred to a solution of cold sucrose buffer. After 48–72 hours in cold sucrose buffer mucus and detritus were removed by means of a delicate aquarelle brush.

The cleaned specimen was immersed 3 to 4 minutes in a 1 per cent aqueous solution of Alcian-green 3BX. Storing in 70 per cent ethanol. The specimen was then checked under a stereomicroscope and appropriate areas photographed at low-semimacroscopic-magnification, using luminar lenses (63 mm) fitted to a Zeiss Ultraphot II camera Microscope.

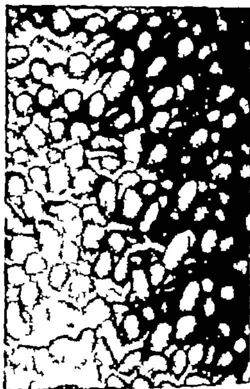


Fig 1 Normal ileum. Uniform, fingerformed villi. Alcian-green × 31

RESULTS

The semimacroscopic surface appearance of a normal ileal mucosa (3) is shown in Fig 1

A detailed description of the semimacroscopic appearance of the mucosal surface of the normal jejunum and ileum is in preparation.

The semimacroscopic investigation of the specimens disclosed a rather uniform picture. The changes, however were more or less pronounced and found in different stages of development. The pathological changes composing the semimacroscopic picture observed in eight specimens were the following

1 *Changes of Villus Appearances*

a) *Slightly enlarged villi.* Thick, short and cylindrical villi. Especially the apical region was enlarged and many villi were club-shaped. The density of goblet cells appeared to be normal (Fig 2). Villi of this configuration were observed in four specimens from three patients (Fig 2 and 4).

b) *Medium enlargement of villi.* Rounded, closely packed distended villi, often flat on the top (Fig 2). On the apex of several villi, small vesicles were observed. The density of goblet cells was judged to be normal. In a few specimens there seemed to be a fusion of villi (Fig 4). Villi of this configuration were observed in seven specimens from five patients.

c) *Greatly enlarged villi.* Large rounded villi at places presenting a picture almost like a cluster of grapes (Fig 3). They were observed in areas with greatly advanced changes (Fig 5). On the apices of these villi, small vesicles might be found too. Density of goblet cells was reduced. These changes were observed in three specimens from two patients.

d) *Absen[ce o]f villi.* In one specimen the surface was completely avillous.

2 *Polypoid Stru[c]tures*

The polypoid structures varied in size as well as shape. Often they were assembled in



F g   Area with both slightly enlarged and moderately *enlarged* VIII. Note [illegible] on the top of some of the moderately enlarged VIII Alcian-green × 27

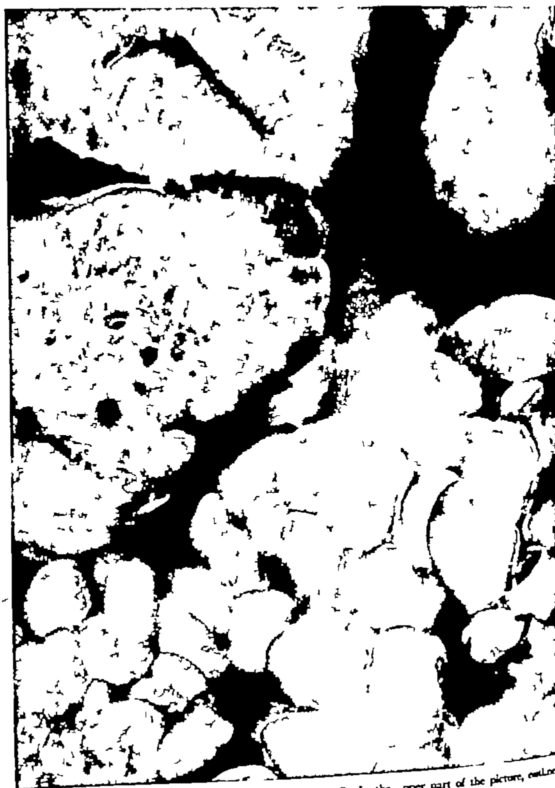


Fig 3 In the lower part of the picture, greatly enlarged ills. In the pper part of the picture, outlines of part of three polypoid structures are seen. Alcian-green 34



Fig. 4 In the centre of the picture, a triangular ulceration. In the surroundings can be observed. A: Polypoid structure covered by nearly normal villi. B Polypoid structure covered by abnormal villi. C Almost avillous polypoid structure. D Villi presumably fusing. E: Slightly enlarged villi. The proximal three polypoid structures (A, B, C) are shown histologically in Fig. 8. Alcian-green × 14

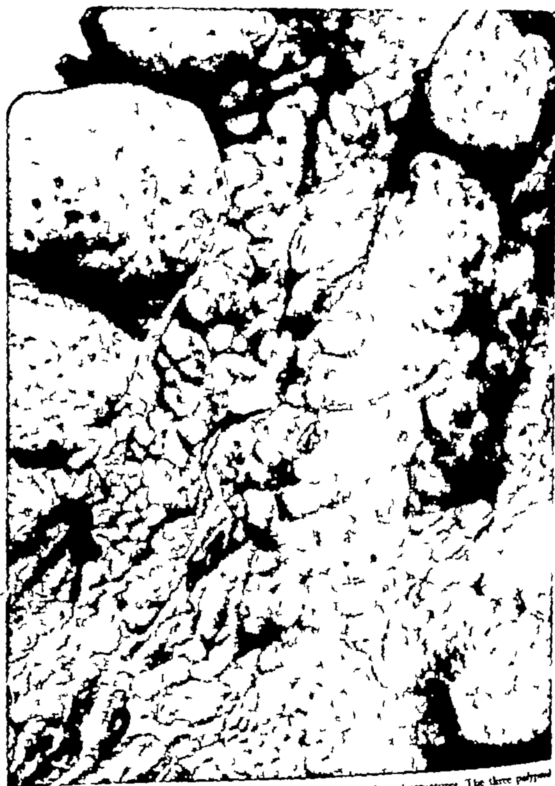


Fig 5 An area with greatly enlarged villi surrounded by avillous polypoid structures. The three polypoid structures to the left in the picture are shown histologically in Fig 9 Alcian-green × 14



Fig 6 Histological section of slightly enlarged villi. The upper part dilated by lymphatics. A vesicle on the top of one of the villi is noticed. PAS-haematoxylin × 100

groups or rows separated by narrow fissures (Fig. 4–5) forming the cobblestone pattern. In most cases their surfaces were avillous (seven specimens) In five specimens polypoid structures were present, covered by enlarged villi some of which might be partly fused In one specimen, polypoid structures covered by almost normal villi were found.

3 *Fissures*

Most often observed as narrow crevices situated between the polypoidlike structures. Semimacroscopically they were difficult to estimate. They were observed in six specimens from five patients.

4 *Ulcerations*

A few ulcerations were deep and penetrating otherwise they were flat-bottomed, sharply demarcated and irregular Often they were confluent. Ulcerations were observed in six specimens from five patients (Fig. 4)

The mucosal surface pattern as here observed is polymorphous. Areas with an almost normal surface may be contiguous to areas showing advanced changes such as polypoid structures, ulcerations and greatly enlarged villi. In the same way all sorts of villus configurations may be observed in the same specimen. The transition between different areas may



Fig 7 Larger magnification of a villus containing dilated lymphatics. In the epithelium a vesicle. PAS-haematoxylin × 200.



Fig 8 Histological section of the three conjoining polypoid structures shown in Fig. 4. Note almost intact epithelium at the bottom of the fissures. One polypoid structure has nearly normal villi, one abnormal villi and one is nearly avillous. PAS-haematoxylin × 20.



Fig 9 Histological section of three avillous polypoid structures. Intact epithelium at the bottom of the fissures. PAS-haematoxylin × 20.

be sharply demarcated or gradually changing, all intermediary stages being represented.

In histological sections, the slightly enlarged villi showed greatly dilated lymphatics, mainly situated centrally in the villi and frequently occupying most of the villus volume. Especially the apical region was affected (Fig 6–7)

In the villi of medium enlargement, dispersed lymphangiectasia, oedema and contents of lymphocytes and plasma cells were observed. Villi were broad short and flat on the top.

The greatly enlarged villi showed in the stroma an inflammatory exudate mainly consisting of focal aggregates of lymphocytes, plasma cells and fibroblasts. Lymphangiectasia and slight oedema were found too.

In all types of villi, the previously mentioned vesicles on the top of the villi were seen in the histological sections. Some of the vesicles contained a few lymphocytes or eosinophils.

The stroma of the polypoid structures (Fig. 8–9) was mainly composed of submucosa which arched up the mucosa and the muscularis mucosae due to swelling, oedema, and inflammation. In the mucosa, dispersed lymphangiectasia and oedema were found especially just below the epithelium. Most of the polypoid structures were avillous, but some were covered by short, broad villi and a few by normal villi. The polypoid structures were separated by fissures which might be deep and ulcerated, as most commonly described. However when the polypoid structures observed semimacroscopically were studied in histological sections it was noticed that the epithelium and mucosa at the bottom of the fissures in several cases were without signs of degeneration or ulceration (Fig 8–9)

DISCUSSION

The most characteristic pathological changes following Crohn's disease are segmentary thickening of the intestinal wall and narrowing of the lumen often resulting in structures. The mucosal changes are ulcerations, fissures and polypoid structures, in some cases producing cobblestone appearance (6, 8, 9 12 13)

The histological pattern may be very complex. It is characterized by (1 5 6, 7 8, 9 10 11 12 13) 1) transmural inflammation, the inflammatory exudate mainly consisting of focal aggregates of lymphocytes, plasma cells, some eosinophils, and polymorphs, 2) thickening of submucosal tissue due to oedema, lymphangiectasia, inflammation and, at later stages, fibrosis, 3) hypertrophy of muscularis mucosae, 4) fissures, 5) ulcerations, 6) polypoid structures, 7) granulomas, 8) neuromatous lesions and 9) pyloric gland metaplasia.

Changes in the mucosal surface pattern and in the villus structure of the small intestine affected by Crohn's disease are not often dealt with in earlier reports.

Concerning the villus changes, *Shiner* (11) described partial or subtotal villus atrophy caused by shortening and thickening of villi by oedema. *Meadows* (5) found villus blunting and *Schmitz* (12) mentioned that villi are distorted. In a recent review *Thayer* (13) mentioned villus atrophy

The heavy changes in the villus structure illustrated in the present paper have apparently not been described in earlier reports.

As stated above, small vesicles are often observed on the apical parts of the enlarged villi. In histological sections they are situated just beneath the epithelium, presumably between the latter and the basal membrane. As far as it is known to the present authors, these vesicles are not mentioned in previous reports dealing with Crohn's disease.

Concerning the histological changes, all specimens in the present material showed changes previously described as typical of Crohn's disease and satisfied the common histopathological requests of this disease. We have therefore concentrated our studies on changes of the mucosal surface structure and, concerning the histological changes, only given a short description relating to the surface studies.

It may be mentioned, that the fissures separating the polypoid structures need not, as usually described, be ulcerated at the bottom. Deep fissures may at the bottom be lined with a tall intact columnar epithelium covering a mucosa without any signs of degenerative activity

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PURIFICATION OF ZYMOGEN GRANULES FROM MONKEY PAROTID GLANDS

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Arneberg, P., Dahl, E. & Hars, R. Purification of zymogen granules from monkey parotid glands. Acta path. microbiol. scand. Sect. A, 83 389–394 1975

A method giving highly purified zymogen granules from *Macaca irus* and *Cercopithecus aethiops* parotid glands is reported. A 0.3 M sucrose medium for homogenization was supplemented with 10 mM tris/HCl, pH 7.3 and 0.1 mM lauric acid to stabilize the fragile monkey zymogen granules. Nuclei and cell debris were sedimented at 150 × g A "crude" zymogen granule fraction was trapped in the 1.0 M sucrose layer of a discontinuous sucrose gradient at 1000 × g Equilibrium centrifugation in a continuous sucrose gradient gave a fraction of zymogen granules at 1.85 M sucrose. Compared to the homogenate this fraction exhibited about two-fold increase in zymogen granule marker whereas mitochondrial marker was reduced to 1/4 and lysosomal marker to 1/2. A low level of contamination from other cell organelles was confirmed by electron microscopic investigation.

Key words Parotid glands zymogen granules purification.

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Zymogen granules (ZG) of high purity can be obtained from homogenates of parotid and pancreatic glands of rodents by differential centrifugation in unbuffered isotone sucrose solutions (1 11) Such methods proved effective in pilot experiments with rat parotid glands in our laboratory but when parotid glands of monkey were used, the ZG were disrupted

The method reported here is based on separations by sedimentation velocity and sedimentation equilibrium in sucrose density gradients. The latter principle has previously been used for the purification of ZG from submandibular glands of guinea pig (2)

MATERIALS AND METHODS

Parotid Glands

Macaca irus or *Cercopithecus aethiops* monkeys were used. Overnight fasted animals were anaesthetized with sodium pentobarbital (Mebumal) and bled from the throat. Within 10 minutes the parotid glands were extirpated, washed in homogenization medium, and dissected free of fat and hilus tissue. The glands were thoroughly minced with scissors in 5 or 10 ml cold homogenization medium. The following procedures were carried out at 0–5 C.

Homogenization

The medium was buffered at pH 7.3 with 10 mM tris/HCl and contained 0.3 M sucrose, 0.2 µg/ml N,N'-diphenyl *p*-phenylenediamine (DPPD) (1) and 0.1 mM lauric acid (13) The DPPD (Koch-Light laboratories) and the lauric acid were added from freshly prepared ethanolic stocks, and

the medium was sonicated for dispersion of the lauric acid before use.

The minced tissue (2–3 g wet weight) was homogenized in 10–15 ml medium/g tissue with a serrated teflon pestle with a clearance of 0.4 mm in a 100-ml glass mortar (Contes Glass Company Vineland, N.J., U.S.A.). From two to five strokes at 1000 rpm were used with the mortar chilled in ice-water. Tissue fragments were removed by filtration through a 265-micron nylon net.

Purification of ZG

All gradients contained 10 mM tris/HCl, pH 7.5. The sucrose concentration in the gradients and the fractionation scheme are outlined in Fig. 1. The homogenate was layered on 12.5 ml 0.55 M sucrose to reduce loss of ZG in the first centrifugation step. The supernatant was applied on a discontinuous sucrose gradient and sedimented through 0.5 M sucrose into a layer containing 1.0 M sucrose and 0.8 per cent glycogen. The glycogen had been purified from Shell fish glycogen (Sigma type II) by the method of *Layter & Schramm* (9) (by courtesy of Dr. A. Hensten-Pettersen).

The final purification was carried out in a continuous sucrose gradient prepared 14 hours previously by layering of 5-ml fractions with sucrose molarities 2.0, 1.9, 1.85, 1.825, 1.8, 1.775, 1.75, 1.7, 1.6, and 1.5. Centrifugation was carried out in a 25/2 SW rotor (Beckman Instrument Co.) at 25×10^3 rpm ($100{,}000 \times g_{max}$) for 2 hours.

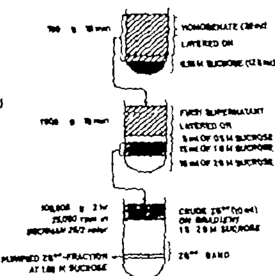


Fig. 1. Purification of zymogen granules by centrifugations in sucrose gradients. All solutions were buffered at pH 7.5 with 10 mM tris/HCl. *The 1.0 M sucrose solution contained 0.8 per cent glycogen. **ZG = Zymogen granules.

Electron Microscopy

A whitish band appeared at about 1.87 (1.78) M sucrose after equilibrium centrifugation. In one experiment, samples from five different levels of this band were fixed in 4 per cent glutaraldehyde at 4° C for 4 hours, washed and postfixed in 1 per cent osmium tetroxide for 1 hour. The fixatives and washings contained also 0.3 M sucrose and 0.1 M sodium phosphate, pH 7.3. The specimens were embedded in Vestopal W. Sections (500–700 Å) were contrasted with uranyl acetate and lead citrate.

Biochemical Assays

Sucrose interfered with the assay of protein and mitochondrial marker. The test samples were therefore adjusted to equal sucrose concentrations and then sonicated at 21 kc/sec and an amplitude of 7 microns for 10 sec (2.5-ml samples) or 15 sec (5-ml samples).

Protein was determined by the method of *Lowry et al.* (8) after treatment of samples in 0.5 N NaOH for 3 hours at 37° C.

ZG-marker was α-amylase (E.C. 3.2.1.1). A semiquantitative test (Sigma Bulletin 700) was used to screen the activity in the continuous sucrose gradient, and subsequent quantitation was carried out by the method of *Jacobsen & Hensten-Pettersen* (5).

The mitochondrial marker was cytochrome c oxidase (E.C. 1.9.3.1). The activity was assayed by the method of *Cooperstein & Lazarow* (3).

The lysosomal marker was acid phosphatase (E.C. 3.1.3.2), which was assayed as described by *Bhoola & Heap* ( ).

RESULTS

Protein recovery and the changes in specific activity of marker enzymes are given in Table 1.

Homogenate. The protein concentration ranged from 5–15 mg per ml in the different experiments and total amounts from 300 to 950 mg protein. M. irus parotid glands gave on an average twice as high amounts as those of C. aethiops. The specific activity of the ZG marker amylase ranged from 100 to 900 units per mg protein.

First supernatant. The total protein was reduced by about one third (range of recovery 53–78 per cent), whereas the specific activity of the three marker enzymes was largely unchanged.

Crude ZG. The fraction contained about

TABLE 1 *Protein Recovery and Changes Activity of Marker Enzymes in the Two Final Steps of Zymogen Granule Purification. Values Are the Mean and Range of 7 Experiments 4 with C. aethiops and 3 with M. irus Parotid Glands. Lysosomal Marker Was Not Assayed i 3 Experiments*

Fraction	Total protein: Per cent of homogenate value	Relative activity of marker enzymes*: Zymogen granules (Amylase)	Mitochondria (Cytochrome c oxidase)	Lysosomes (Acid phosphatase)
Crude zymogen granules	6.8 (3.2–12.8)	1.20 (0.81–1.63)	0.39 (0.10–0.78)	0.42 (0.17–0.71)
Purified zymogen granules	1 1 (0 5–3.0)	2.28 (1.55–3.75)	0.25 (0.05–0.54)	0.47 (0.18–0.76)

Activity per mg protein in fraction/activity per mg protein in homogenate

7 per cent of the homogenate protein. The specific activity of ZG marker was somewhat increased, whereas those of mitochondrial and lysosomal markers were reduced by about one half

Equilibrium centrifugation A broad whitish band was found at about 1.87–1 78 M sucrose

The morphologic evaluation indicated that ZG accounted for more than 95 per cent of cell organelles throughout the entire depth of this band (Figs. 3a, b, c) A few mitochondria were identified (Fig 3b, left) and some nuclei observed at the highest sucrose density However all ZG were not equally well preserved. Partially extracted granules were observed (Fig. 3d, right) as well as empty membrane vesicles (Figs. 3a, c)

A size distribution analysis of the ZG from different levels of the band demonstrated a predominance of small granules in its upper part (Table 2)

Biochemical analyses showed that the whitish ZG-band at 1.87–1 78 M sucrose contained 60–80 per cent of the amylase activity applied. The highest specific activity of this ZG-marker was found in the lower half of the band (Fig. 2)

Purified ZG Based on amylase screening test, this fraction (4.5–5 ml) was selected at about 1 85 M sucrose in the gradient. It contained 0.5 to 3 per cent of total homogenate protein. A pair of glands from the *M irus* monkey gave as an average, 9 mg protein in this fraction, compared with about 4.5 mg from *C aethiops* corresponding well with the difference in total glandular protein

In the final step, from crude ZG to purified ZG (Fig 1) the specific activity of the ZG-marker increased 1.5–2 times, and the

TABLE 2 *Size Distribution Analysis of Zymogen Granule o Electron Micrographs. A Comparison of Samples f om the Lower Middle and Upper Part of the Zymogen Granule Band*

Fraction No.	Sucrose molarity of fraction	Percentage of total granules with diameters exceeding 0.75 µ	1.00 µ	1.25 µ	1.5 µ
1	1.87	79.9	42.9	18.7	5.0
3	1.84	72.9	33.3	12.1	2.4
5	1.80	51.6	14 1	2.4	0.1

The largest granule diameter was grouped by means of concentric circles of diameter 0.9 1.2, 1.5 and 1 8 mm on micrographs (× 12,000) The granules on 10 micrographs from each fraction (970–1080 granules) were measured.

DISTRIBUTION OF PROTEIN AND MARKER ENZYME ACTIVITIES

-o- ZYMOGEN GRANULE MARKER
▨ MITOCHONDRIAL MARKER
■ LYSOSOMAL MARKER
-▲- PROTEIN (mg/ml)

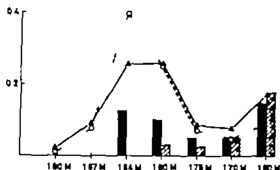


Fig 2 Purification of zymogen granules in 1.5–2.0 M sucrose gradient. Zymogen granule marker 5×10^3 amylase units/ml. Mitochondrial marker Δ $OD_{[illegible]}$ in cytochrome *c* oxidase test. Lysosomal marker $OD_{[illegible]}$ in acid phosphatase test.

corresponding value for the mitochondrial marker was reduced to about one half where as that of lysosomal marker was not further reduced. Comparison of amylase activity and cytochrome *c* oxidase activity indicated that the ratio ZG *versus* mitochondria increased five times or more during purification. The corresponding amylase/acid phosphatase ratio increased about four times (Table 1)

DISCUSSION

The previously published methods led to rupture or extraction of the monkey ZG dur

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Fig 3 Electron micrographs of samples from the zymogen granule band at 1 87–1 78 M sucrose (a, b, c × 6000, d × 30 000) a) Lowest part of the band (1.87 M sucrose) showing large zymogen granules (ZG) and some membrane material b) Middle part of the band (1.84 M sucrose) showing fairly large ZG and a mitochondrion (m) c) Upper part of the band (1.80 M sucrose) showing small ZG and some empty membrane vesicles. d) High magnification of almost completely extracted ZG (arrows)

ing homogenization and resuspension. Elimination of most connective tissue before homogenization, addition to the medium of tris buffer and lauric acid avoiding resuspension of ZG in the procedure improved the yield with monkey parotid glands. Hypertone sucrose solutions, that rupture ZG of the rat parotid gland (6) were tolerated by the monkey granules. A separation of ZG from lysosomes and mitochondria was achieved by their higher sedimentation velocity in a discontinuous sucrose gradient (Fig 1 Table 1) The glycogen added to this gradient to prevent aggregation of subcellular particles (10) appeared to improve the separation, possibly by increasing the viscosity

After equilibrium centrifugation, the fraction at 1.85 M sucrose in the lower half of the ZG band exhibited the highest specific activity of amylase, and was selected as purified ZG (Figs. 1 2) As the morphologic evaluation showed low levels of contamination with other cell organelles throughout the whole depth of this band (Figs. 3a, b, c) the observed amylase distribution may reflect accumulation of partially extracted ZG in its upper part. The smaller size of these granules could be an artefact caused by shrinkage in the strongly hypertone sucrose solution (Table 2)

The presented method gave about 1 per cent of glandular protein as purified ZG (Table 1) This is comparable to the recovery of guinea-pig pancreatic ZG by differential centrifugation in isotone sucrose (11) The latter principle was not useful with monkey parotid glands, but gave about 10 per cent recovery with the rat parotid (1) However all methods result in a considerable loss of ZG The ZG occupy about one third of the parotid acinar cell volume in the fasting rabbit (4) and have been estimated to approximately one half of total protein in the rat parotid (12) This estimate corresponds fairly well with the about two-fold increase in specific amylase activity observed during isolation of ZG from various serous glands (1 11) (Table 1)

Lysosomes exhibit a similar density to ZG in sucrose solutions, and mitochondria have a slightly lower density (2) (Fig. 2) The purification was therefore followed by marker enzymes for these organelles (Table 1) Mitochondria account for about 6 per cent of total protein in the rat parotid gland (1) Assuming a similar percentage in monkeys, the residual activity of mitochondrial marker indicated about 1.5 per cent of mitochondrial protein in the purified ZG fraction. Contamination by lysosomes is assumed to be lower in spite of the somewhat less efficient elimination of this marker (Table 1) because there is considerably less lysosomes than mitochondria in glandular tissue (7)

In conclusion, electron microscopic observations as well as the activities of marker enzymes indicate that a highly purified fraction of monkey ZG is obtained by the presented method.

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ANTIHYPERTENSIVE AND HYPERTENSIVE EFFECTS OF THE KIDNEY

Elucidated by Treatment with Medullary Transplants and with Blockade either of the Reninangiotensin-System or of the Prostaglandin Biosynthesis

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Manthorpe, T. Antihypertensive and hypertensive effects of the kidney. Elucidated by treatment with medullary transplants and with blockade either of the reninangiotensin-system or of the prostaglandin biosynthesis. Acta path. microbiol. scand. Sect. A, 83 395-405 1975.

After subcutaneous autotransplantation of renal medulla either from normal donors or from the ischaemic kidney of renal two-kidney hypertensive rats, the blood pressure of the renal two-kidney hypertensive recipients was lowered—but not to normal levels. After i.v. injection of the converting enzyme inhibitor SQ 20,881 the blood pressure was further decreased to, or close to, normal blood pressure. A complete normalization of the blood pressure was obtained by the combined treatment with medullary transplants and infusions of the angiotensin II inhibitor Saralasin. The difference between the effects of these two blockers was probably caused by differences in the doses used. Administration of indomethacin, an inhibitor of the prostaglandin biosynthesis, to renal two-kidney hypertensive rats with or without renomedullary transplants failed to provoke a rise in blood pressure. This indicates that the anti-hypertensive activity of renomedullary transplants is not due to the group of prostaglandins, the synthesis of which is inhibited by indomethacin, and furthermore that these prostaglandins are not of importance to the blood pressure level in renal two-kidney hypertensive rats.

Key words: Renal hypertension; renomedullary transplants; blockers of the renin system; indomethacin.

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The antihypertensive action of normal kidneys has been localized to the renal medulla (for literature see *Muirhead* 1974). In a previous investigation (*Manthorpe* 1973) it was confirmed that, following subcutaneous autotransplantation of normal renal medulla to renal hypertensive rats, the blood pressure would remain depressed for a long period of time. In addition it was shown that transplantation of medulla from the *ischaemic* kidney of two-kidney hypertensive donors produced a similar depression of the blood pressure, whereas medulla from the *untouched* kidney had little or no effect.

The primary aim of the present study was to test whether these findings, which were based on a limited number of animals, could be reproduced in a larger number of animals. It was further investigated whether the lowered, but not normalized, blood pressure of renal hypertensive rats with medullary

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transplants could be further decreased by blockers of the renin system. In order to elucidate whether the blood pressure lowering effect of renal medullary transplants might be due to release of prostaglandins, the effect of indomethacin, a blocker of prostaglandin biosynthesis, was studied.

MATERIAL AND METHODS

Animals Inbred female SPF Lister rats, weighing about 170 g, were fed commercial chow (Rostock pills containing 0.3 per cent NaCl) and given tap water *ad libitum* *Indirect blood pressure* measurements in unanaesthetized animals were obtained by the tail plethysmographic method (*Williams et al.* 1939) Each measurement was obtained by taking the average of 5 consecutive readings. For direct blood pressure measurements, catheters were implanted according to the method of *Popovic & Popovic* (1960) as modified by *Berg & Nielsen* (1973A) Systolic arterial pressure was recorded using a Tybjerg Hansen capacitance transducer and a Servogor 511 recorder some of the rats were pretreated with 5 μg ergotamine tartrate. *Hypertension* was provoked by placing a 0.20 mm silver clip on the left renal artery (*Wilson & Byrom* 1939) the right kidney being left untouched the operation was performed under light ether anaesthesia on rats pretreated with 22,500 I.U Dipenicillin LEO® i.m. per 100 g body weight (b.w) Control rats (non-transplanted) had the clip applied at the same time as those to be transplanted later on and were kept in the same cages. *Transplantations* were performed following the method of *Muirhead et al.* (1970) as modified by *Manthorpe* (1973) The transplanted amount of renal medulla was approximately 0.55 g from 6 normal 0.25 g from 4 ischaemic, and 0.35 g from 4 untouched kidneys, respectively *Histological investigations* When the rats were sacrificed the transplants were removed, fixed in 4 per cent buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin-eosin, PAS and van Gieson-Hansen. The converting enzyme inhibitor was the nonapeptide Pyr Trp-Pro-Arg Pro-Glu-Ile-Pro-Pro, designated SQ 20,881 (E. R. Squibb & Sons, New York) given as a single dose of 2 mg per kg b.w i.v The angiotensin II inhibitor was the 1-Sar-8-Ala-angiotensin II (Sar Arg-Val Tyr Val-His-Pro-Ala, designated Saralasin, the Norwich Pharm. Corp., New York) which was infused in dose of 20–80 mg per kg b.w per hour A Braun perfusor was used for the infusion. Indomethacin was given either orally (10 mg per kg b.w in 175 mM $NaHCO_3$ solution) by gastric tube or intravenously by injection of 2.0 mg per kg b.w followed by infusion of 2 mg per kg b.w per hour = 1 ml per kg b.w. per hour This dose was greater than the dose used by *Leyssac et al.* (in press) in their experiments on the effect of indomethacin blockade of renal PGE-synthesis on total renal and tubular function and on plasma renin concentration in hydropenic rats. Their dose gave a plasma concentration of indomethacin of 13.6 ± 2.5 μg ml^{-1} which was stable for hours, and the prostaglandin E_2 dropped in renal venous blood from 216 ± 35 pg ml^{-1} to 83 ± 4 pg ml^{-1} A modified Ringer's solution was used as solvent. Blockers of the renin system and indomethacin were given 2½-3 months after the transplantation with medullary tissue either from ischaemic or normal kidneys. *Statistical evaluation* The differences between the groups were analysed using the "Student's" t-distribution or the Wilcoxon Rank Sum test. Differences were considered significant if $p < 0.05$.

RESULTS

Effect of Transplantation of Renal Medulla to Renal Two-Kidney Hypertensive Rats

Three groups of unanaesthetized rats were studied Group 1) renal two-kidney hypertensive control rats without transplantation group 2) renal two-kidney hypertensive rats transplanted with renal medulla from the *ischaemic* kidneys of renal two-kidney hypertensive donors group 3) renal two-kidney hypertensive rats transplanted with medulla from the *untouched* kidneys of the donor rats. The data, which include 3 4 and 4 animals from the previous study (*Manthorpe* 1973) in groups 1 2 and 3 *respectively* are given in Fig 1

Before the transplantation the systolic arterial pressure was equal in all three groups 195 ± 2 mm Hg 195 ± 1 mm Hg and 193 ± 2 mm Hg respectively It appears that blood pressure did not change for more than 8 days either in the control rats (group 1) or in rats transplanted with medulla from untouched kidneys (group 3) In contrast, the blood pressure in rats transplanted with medulla from the ischaemic kidneys (group 2) was significantly lower than that in rats in the other groups. During the first 23 days after transplantation, the blood pressure mean in animals in group 2 was 178 ± * mm Hg

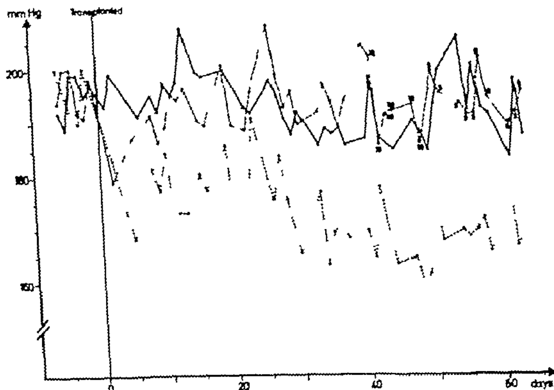


Fig. 1. Indirectly measured systolic arterial pressure of renal two-kidney hypertensive rats. —— indicates non-transplanted controls (n = 8). - - - indicates rats transplanted with medulla from the untouched kidneys (n = 11 at the start of the experiment) ····· indicates rats transplanted with medulla from the ischaemic kidneys (n = 10). Time zero indicates day of transplantation.

as compared with that in animals in group 1 (196 ± 2 mm Hg) and group 3 (192 ± 2 mm Hg) respectively ($p < 0.001$). In the following 5–6 weeks, this difference became even more pronounced: the blood pressure mean in rats transplanted with medulla from ischaemic kidneys (group 2) decreased to 169 ± 1 mm Hg, while that of rats transplanted with medulla from untouched kidneys (group 3) remained at 193 ± 1 mm Hg, that of the control rats (group 1) remaining at 190 ± 1 mm Hg.

2½–3 months after transplantation, the rats in group 2 (ischaemic medulla) showed great variations in blood pressure, both from day to day and within the same day. (Prior to this, the rats in group 3 (untouched medulla) had been used for other purposes, as described below.) On several occasions it was observed that a rat might have a blood pressure between 150 and 170 mm Hg in the morning and that it was above 200 mm Hg after the implantation of the catheters, both determinations being performed by the indirect method. In all cases there was fairly good agreement with the indirectly measured blood pressure and the blood pressure measured directly shortly afterwards.

Effect of Transplantation of Medulla from Normal Kidneys to Renal Hypertensive Rats 63 Days after the Transplantation of Medulla from the Untouched Kidneys

Five of the rats which were transplanted with medulla from untouched kidneys survived for a period sufficiently long to allow transplantation of normal renal medulla at

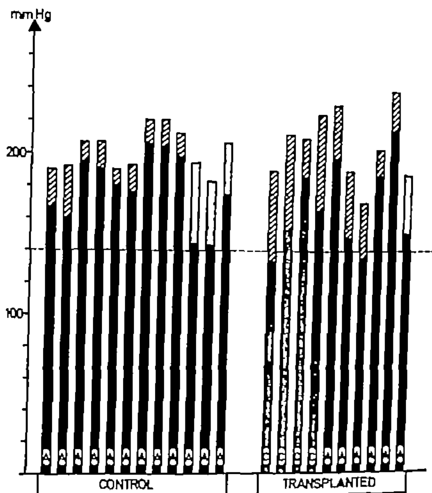


Fig 2 Shows the effect of the *converting enzyme inhibitor* (SQ 20,881) on the directly measured blood pressure of renal two-kidney hypertensive rats. Group 1 rats were non-transplanted controls, group 2 rats were transplanted with renal medulla. Each column represents an individual experiment. The top edge of the columns indicates the systolic arterial pressure level in mm Hg immediately before the injection the black columns show the lowest blood pressure obtained after the injection. Hatched and grey columns indicate the drop in blood pressure. Grey columns indicate pre-treatment with indomethacin. The horizontal broken line indicates the upper limit of normal blood pressure. The letters (C) and (A) indicate measurements in conscious and anaesthetized rats, respectively

later stages. One of these 5 rats died within the first week after the second transplantation. The mean values of the blood pressure in the remaining 4 rats are given in Table 1. It can be seen that there was no significant difference between the blood pressure mean before and after the transplantation of untouched medulla ($p>0.5$) whereas there was a significant lowering of the blood pressure following the transplantation of normal renal medulla ($p<0.001$)

Histology

The transplants consisted of living tubular and living interstitial cells in a highly vascularized amorphous and hyalinized ground substance in addition there were many haemosiderin containing macrophages. There were no signs of rejection. The renomedullary transplants were surrounded by connective tissue. In the light microscope there was apparently no gross difference between the

TABLE 1 *Systolic Arterial Blood Pressure in Goldblatt Two-Kidney Hypertensive Rats before and after Transplantation of Medulla from Untouched Kidneys and after Second Transplantation of Normal Renal Medulla*

	Before the transplantations	*After the first transplantation* Medulla from untouched kidneys	*After the second transplantation* Medulla from normal kidneys
Days	-6 0	26 – 63	69 – 126
BP	191 ± 3	187 ± 2*	174 ± 1‡
n	4	4	4

BP = systolic arterial blood pressure in mm Hg expressed as mean ± S.E.M.
* = $p > 0.5$ compared with "before the transplantations"
‡ = $p < 0.001$ compared with "after the first transplantation"
n = number of rats.

transplants from the three types of donor kidneys (normal, ischaemic and untouched)

Effect of Blockers of the Renin-Angiotensin System

Two groups were investigated group 1) renal two-kidney hypertensive control rats and group 2) renal two-kidney hypertensive rats transplanted with renal medulla either from ischaemic or normal kidneys. Since there was no difference in response of those transplanted with ischaemic and those transplanted with normal medulla, the results obtained in these rats are pooled. Following

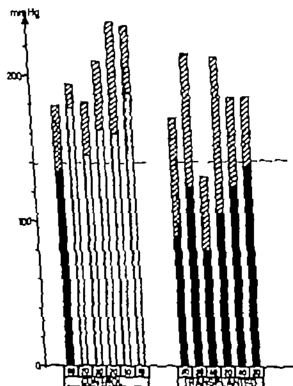


Fig 3 Shows the effect of *angiotensin II inhibitor* (Saralasin) on the directly measured systolic arterial pressure of conscious renal two-kidney hypertensive control rats and renal two-kidney hypertensive rats with medullary transplants either from ischaemic or normal kidneys. Each column represents an individual experiment. Grey columns represent data from *Bing & Nielsen (1978)* black columns represent personal experiments. The top edge of the columns indicates the blood pressure level in mm Hg immediately before the infusion. The black and the grey columns indicate the lowest blood pressure level obtained during the infusion. The number below the column indicates the dose infused in $\mu g \times kg\ b.w.^{-1} \times min^{-1}$. The horizontal broken line indicates the upper limit of normal blood pressure.

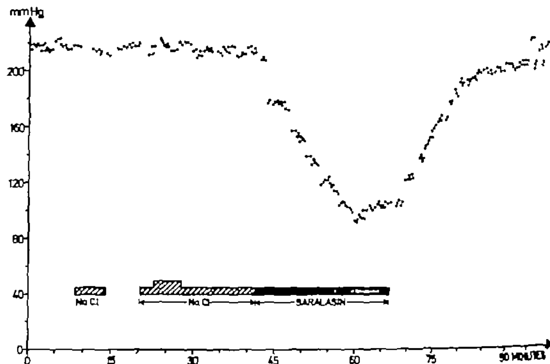


Fig 4 Shows the effect on directly measured systolic arterial pressure of a renal two-kidney hypertensive conscious rat during the infusion of 0.9 per cent saline (4–8 µl × min) and of Saralasin (20 µg × kg b.w × min = 4 µl × min⁻¹) The rat was previously transplanted, first with medulla from untouched kidneys and later on with medulla from normal kidneys.

the administration of the *converting enzyme inhibitor* (SQ 20,881) there was a fall in blood pressure in the non-transplanted (group 1) as well as in the transplanted rats (group 2) (Fig 2) but the drop in pressure was significantly greater in the transplanted rats than in rats in the control group (Wilcoxon Rank Sum test $0.01 < p < 0.02$) The control group includes 3 rats to which indomethacin was administered the fall in blood pressure in these was more pronounced than in the other control rats. If the data concerning these indomethacin treated rats are excluded, the difference in pressure response of controls and transplanted rats becomes even more significant ($p < 0.002$)

Infusion of the *angiotensin II inhibitor* (Saralasin) provoked an even more pronounced fall in blood pressure both in the transplanted and in the non-transplanted control group than after injection of SQ 20,881 the difference was significant (Wilcoxon Rank Sum test $p = 0.02$ for the transplanted groups, and $p = 0.01$ for the control groups, when the indomethacin pretreated rats were excluded) Due to shortage of Saralasin all results obtained in a study of conscious, long term hypertensive rats presented by *Bing & Nielsen* (1975B) were included in the present non-transplanted control group (Fig. 3) During infusion of Saralasin, the transplanted rats again showed a fall in pressure which was significantly greater than that in control rats ($p = 0.02$) (Fig 3) Fig 4 shows a typical experiment. The systolic arterial pressure remained unchanged during the infusion of 0.9 per cent saline whereas there was a gradual depression to normal blood pressure values within the first 20 minutes during the infusion of Saralasin. The blood pressure remained stable and normal until the infusion was interrupted when it gradually rose to the pre-infusion level during the next 25 minutes. About 5–10 minutes after the blood pressure had regained the hypertensive level, the rat had several con-

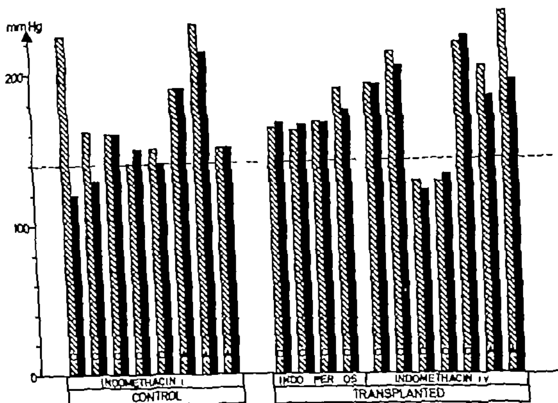


Fig 3 Shows the effect of *indomethacin* on systolic arterial pressure of renal two-kidney hypertensive rats group 1 rats were non-transplanted controls group 2 rats were transplanted with renal medulla Hatched columns indicate blood pressure before indomethacin treatment black columns indicate blood pressure after the administration of indomethacin.

vulsions, though it until then apparently had been in good shape sleeping calmly without anaesthesia.

Effect of Indomethacin on the Blood Pressure

Fig 3 shows the changes in systolic arterial pressure following administration of indomethacin, either orally or intravenously In rats given indomethacin orally the blood pressure value prior to treatment represents the average of the blood pressure values obtained by indirect measurements on the last 12 occasions during a two-week period the blood pressure value after *treatment* is the pressure indirectly measured 45–60 minutes after administration of indomethacin by gastric tube In rats given indomethacin intravenously the blood pressure was measured directly In 5 of the 11 transplanted rats, the blood pressure was significantly lower at the time of indomethacin treatment than before the transplantation. The effect of indomethacin was tested in the remaining 6 transplanted rats at a time when the blood pressure—after a temporary fall—had regained values close to those measured before the transplantation. There was no consistent effect of indomethacin either in the control group or in the group transplanted with medulla from ischaemic or normal kidneys.

DISCUSSION

It is generally assumed that the presence of an untouched kidney is the reason why hypertension in two-kidney hypertension is more

benign than that in one-kidney hypertension, because of the hypertension often is seen to aggravate after removal of the kidney concerned (*Goldblatt et al* 1934) According to this view the effect of transplantation of renal medulla from untouched kidneys might be expected to be greater than that of transplantation of renal medulla from ischaemic kidneys. However quite the opposite has been shown (*Manthorpe* 1973) The present study confirms the findings in a larger material in which transplantation of medulla from ischaemic kidneys gave a depression of the blood pressure in 10 out of 10 recipients similar to that found in the present study and in that by *Muirhead et al.* (1970) after transplantation of medulla from normal kidneys medulla from untouched kidneys had no effect in 9 recipients, and only a slight effect in 2 out of 11 recipients.

The fall in blood pressure seen in 3 out of 4 recipients after the second transplantation of normal renal medulla 63 days after they had been transplanted with medulla from untouched kidneys (Table 1) indicates that the recipients at that time actually were able to react with a lowering of the blood pressure if the transplants possessed a blood pressure lowering activity

Why such activity is absent in transplanted medulla from untouched kidneys remains to be explained. The aggravation of the hypertension after removal of the untouched kidney might, however simply be due to loss of its excretory function rather than to loss of a secretory (antihypertensive) function. It may also seem strange that transplantation of medulla from ischaemic kidneys is able to lower the blood pressure since the recipient has already one ischaemic kidney This effect might be due to a quantitative difference after transplantation, the recipient has an excess of medullary tissue from the 4 donor kidneys in addition to its own. Another possible, but purely speculative explanation could be that the presence of the clip on the renal artery inhibits an antihypertensive function of the ischaemic kidney and that this inhibition disappears from the transplanted tissue. A combination of these explanations is also possible.

The observations that transplantation of renal medulla (*Muirhead et al* 1970, and *Manthorpe* 1973) as well as intravenous administration of angiotensin II inhibitors (*Bing & Poulsen* 1970, *Pals et al.* 1971 *Brunner et al* 1971 and *Bing & Nielsen* 1973) lowers the blood pressure—although not to normal values—in renal two-kidney hypertensive rats made it attractive to investigate the effect of the transplantation and angiotensin inhibitors in the same rats. The transplanted rats were given either SQ 20,881 or Saralasin (Figs. 2–4) In all of the transplanted rats, but only in one of the non-transplanted control rats, the blood pressure was restored to normal by Saralasin as long as the infusion was continued (Figs. 3 and 4) Saralasin had a greater blood pressure lowering effect than SQ 20,881 which produced normal blood pressure values in only 2 of the transplanted rats and in none of the control animals. This difference is probably due to the transitory effect to be obtained after the single injection of SQ 20,881 rather than to the effect obtained by continuous infusion of Saralasin (Bing, personal communication)

Muirhead et al. (1974) found that SQ 20,881 prevented the development of malignant, but not of benign, Goldblatt one-kidney hypertension in rabbits if the treatment was initiated at the time of operation (application of clip and contralateral nephrectomy) Renomedullary autotransplants combined with the SQ 20,881 injections delayed the development of benign hypertension but did not prevent it (*Muirhead et al.* 1974) The apparent discrepancy between their results and those obtained in the present study might be due to the fact that Muirhead investigated the *acute* phase of Goldblatt *one-kidney* hypertension (in rabbits) while the *chronic* phase of Goldblatt *two-kidney* hypertension (in rats) was investigated in the present study However this difference in reaction to inhibitors of the renin-angiotensin system is in accordance with previous findings by

other investigators. Thus, *Brunner et al.* (1971) and *Pals et al.* (1971) found that angiotensin was without significant importance to the blood pressure level in chronic *one* kidney hypertension, but was significantly involved in the chronic *two*-kidney hypertension in rats. *Romero et al.* (1973) reported that this also was true of rabbits, whereas *Bing & Nielsen* (1973B) found that the blood pressure in chronic *one* kidney hypertensive rats would be lowered during Saralasin infusion, but to a lesser degree than in *two*-kidney hypertensive rats.

Even though medullary transplantation (from ischaemic as well as from normal kidneys) was effective in that it lowered the arterial pressure this effect is temporary. About 2 3 months after transplantation, the arterial pressure had usually returned to, or close to the pre-transplantation hypertensive level (*Manthorpe* 1973). This fact explains why the arterial pressure level in transplanted rats and in non-transplanted control animals was equally high at the time of treatment, using blockers of the renin system and indomethacin. However in spite of this secondary increase in arterial pressure, the effect of blockers of the renin system was more pronounced in the transplanted group than in the non-transplanted controls. This observation suggests that some function of the transplants is remaining at this time. It follows that the secondary increase in blood pressure to occur after the temporary blood pressure lowering effect of transplantation is less likely to be due to complete necrosis of the component(s) in the transplanted tissue which acted antihypertensively. The difference between transplanted and non-transplanted rats in their response to these blockers rather suggests that the transplants somehow brought about that the recipients became more sensitive to the blockers than the controls.

The normalization of the blood pressure during Saralasin treatment of transplanted rats seems to indicate that two different and *addit.* mechanisms are responsible for the elevation of the blood pressure following application of the renal artery clip in two-kidney hypertension firstly the renin-angiotensin system and secondly the failure of the ischaemic and/or of the untouched renal medulla to produce the antihypertensive factor(s).

The antihypertensive effect of the renal medulla has been attributed either to release of prostaglandins (*Strong et al.* 1966, *Daniels et al.* 1967 and *Muirhead et al.* 1972) or to release of a neutral lipid (*Muirhead et al.* 1964 and 1968) or finally to release of a phospholipid renin pre inhibitor (*Sen et al.* 1968) from the renomedullary interstitial cells. The renomedullary interstitial cells contain osmophilic lipid droplets, the number of which is highest in normal medulla, lower in the medulla from ischaemic kidneys, still lower in the medulla from the untouched kidneys (*Ishii & Tobian* 1969) and lowest in medulla from "post-salt" hypertensive rats with only one kidney (*Tobian et al.* 1969). In spite of this low number of lipid droplets, the medulla from "post-salt" hypertensive donors depressed the blood pressure after transplantation to "post-salt" hypertensive recipients more than normal medulla (*Tobian & Azar* 1971), a fact which raises the question how an observed change in the number of lipid droplets is to be interpreted. The inhibitor of the prostaglandin biosynthesis, indomethacin, was given to transplanted and non-transplanted renal hypertensive rats in an attempt to single out the lipid substance that could possibly be the active factor. If prostaglandins were responsible for the antihypertensive effect of renomedullary transplants, a *rise* in blood pressure might be expected when indomethacin was given, provided that indomethacin in concentrations sufficient to inhibit the prostaglandin synthetase, reaches the transplants and this seems likely since the transplants were well vascularized. However in the present study there was no significant effect in any of the two groups: a blood pressure *fall* was occasionally observed in both groups (Fig. 5). A marked fall in blood pressure following indomethacin, 10 mg per kg b.w. given orally twice at a 24-hour interval, has been observed

previously (unpublished observations) in these cases it was accompanied by gastro-intestinal bleeding and intestinal perforations —complications to indomethacin treatment which were fatal. This side effect has also been seen by *Brodie et al.* (1970) Such complications to indomethacin treatment could hardly be an explanation of the occasional fall in blood pressure seen in the present experiment because blood pressures were measured very briefly after the indomethacin administration. It can thus be concluded that the blood pressure lowering effect of the transplants is not due to an effect of those prostaglandins, the synthesis of which is inhibited by indomethacin.

The study has been supported by grants from the *Danish Heart Foundation.* I am further indebted to Dr *A Castellion* The Norwich Pharmacal Co. and to Dr *F Giarusso* the Squibb Institute for the kind donation (to Dr J Bing) of Saralasin and SQ 20,881 respectively and to *Løvens Kemiske Fabrikker* and *A/S Dumex* who kindly donated penicillin and indomethacin, respectively I am very grateful to Dr Poul Faarup who gave useful advice in matters concerning the evaluation of the renomedullary transplants. The Tybjerg Hansen transducer and the Servogor recorder were given to the Institute by grants from *The Danish Foundation for the Advancement of Medical Science Nordisk Insulinfond* and the *Legacy of Miss P A Brandt*

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EXPERIMENTAL RENAL CANDIDIASIS IN MICE AND GUINEA PIGS

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Winblad, B. Experimental renal candidiasis in *mice and guinea pigs*. *Acta path. microbiol. scand.* Sect. A, 83 406–414 1975

As a part of an experimental study of the spread of urogenital infections, male guinea pigs and mice were intravenously injected with a sublethal dose of *Candida albicans* in a long-time experiment The kidney was the organ of maximum infection. Spread in the kidney was observed from the cortical and glomerular capillaries, where the injected yeast cells first lodged, but after pseudomycelial transformation penetrated into the Bowman's space and into the lumen of the tubuli. Guinea pigs recovered from the infection. In mice the renal candidiasis progressed and two types of the disease could be distinguished an acute type with cortical abscesses and a chronic type with partly necrotic tips of the pyramids and adhering fungal masses in the pelves. A similar picture has been observed in man. The pathogenesis of renal candidiasis seems to resemble that of renal tuberculosis.

Key words Candidiasis renal *Candida albicans* spread of infection guinea pigs mice scanning electron microscopy

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Candida albicans has been recovered with increasing frequency from human urine (*Schonebeck* 1972, *Blazevic et al* 1972 *Ansehn* 1974) Factors which favour the growth of yeast like fungi are general debility treatment with immunosuppressive drugs or broad-spectrum antibiotics. Predisposing local factors in the urinary tract are indwelling catheter stenosis and glycosuria. We have described some cases in man where the renal candidiasis had disastrous consequences (*Schonebeck et al 1970 Schönebeck & Winblad* 1971 *Schönebeck et al.* 1972)

Experimental *Candida albicans* infections have been used for the evaluation of anti mycotic drugs and this has led to increased interest in the mechanism of pathogenesis of the disease The kidney has been found to be the organ of maximal involvement in experimental infection with *Candida albicans* in mice (*Adriano & Schoenes* 1955 *Blyth* 1958, *Louria et al.* 1960 *Hurley & Winner* 1963) and guinea pigs (*Redaelli* 1924 *Facente et al.* 1952 *Winner* 1960 *Airman & Rybacka* 1971)

The purpose of the present paper was to study the course of renal lesions in candidiasis in mice and guinea pigs after intravenous injection of sublethal doses of fungi.

MATERIAL AND METHODS

Animals Male white Swiss mice weighing approx. 20 g and male guinea pigs weighing approx. 550 g were employed They were housed in plastic cages and had free access to food and water.

TABLE 1 *Virulence for Mice and Guinea Pigs of Candida albicans SI and H 12*

Number of yeast cells injected intravenously (millions)	Mortality rate of the mice within 20 days after infection	Mean survival time for mice (days)	Mortality rate of the guinea pigs within 20 days after infection	Mean survival time for guinea pigs (days)
50.0	5/5*	1.0	3/3*	2.0
10.0	5/5	4.8	3/3	6.3
2.0	5/5	5.8	2/3‡	10.7
0.4	5/5	12.0	1/3‡	14.7
0.08	1/5‡	18.8	0/3	

* numerator: number of animals succumbing; denominator: total number of animals in each group.
‡ for the animals which were still alive on the 20th day a survival time of 20 days has been calculated.

Inoculum Candida albicans strain H 12* was harvested in physiological saline from a 24-hour Sabouraud's glucose agar slant incubated at 30° C. In mice 0.2 ml of the inoculum was injected into lateral tail vein and, in guinea pigs, into the right jugular vein. To determine a suitable sublethal dose for long-time experiment, different amounts of yeast cells were injected (Table 1). The different doses were obtained by dilution of the yeast suspension in physiological saline 1:5, 1:25 and so on. Viable counts were made on 0.1 ml from a dilution 1:100,000. For each inoculum dosage 5 mice and 3 guinea pigs were used.

In the long-time experiment 50 mice and 20 guinea pigs were used. Regarding the survival time of the animals, as seen in Table 1, a suitable yeast cell inoculum dose was considered to be 0.8 × 10⁶ for mice and 0.4 × 10⁶ for guinea pigs.

Morphological studies Two mice were sacrificed at 1, 2, 4, 8 and 24 hours after inoculation, then every 3rd day up to the 40th day and, thereafter every 10th day. Based upon the number of animals succumbing to infection, the course of infection could be followed up to the 50th day. One guinea pig was sacrificed after 8 hours, then after 1, 2, 3, 4, 6, 8, 10, 12 and 15 days and thereafter every 5th day up to the 50th day. Kidneys, brain, heart, lungs, liver, spleen and, in the guinea pigs, pelvic and scrotal genital organs, were fixed in neutral formalin. Paraffin and plastic embedded sections were stained with haematoxylin-eosin, periodic acid-Schiff and with Grocott silver methenamine.

For scanning electron microscopy (SEM) formalin fixed material from mice kidneys was freeze-dried and coated with gold under continuous rotation and tilting of the specimens in a vacuum of 10⁻⁵ Torr. The preparations were then examined in Cambridge Stereoscan S4 Scanning Electron Microscope operated at 5–20 kV.

* Kindly supplied by Dr H. J. Scholer, Hoffmann-LaRoche, Basel, Switzerland.

RESULTS

Mice Although the inoculum dose of 0.8 × 10⁶ *Candida albicans* cells was considered sublethal, 3 mice died due to Candida infection during the first week. 5 animals died during the second week and another 3 animals during the following weeks.

Morphologically two types of disease, the acute and the chronic, were distinguished. All mice sacrificed within ten days after inoculation showed an *acute type of disease* characterized by development of numerous cortical renal abscesses and by abscesses of the heart and brain, but without any pathological changes of the renal pelvis. In animals surviving beyond ten days, the *chronic type of disease* occurred. Here no abscesses were seen in any organs. To a various degree, one or both kidneys were hydronephrotic with the pelvis and the upper part of the ureter filled with fungal growth. In some of the animals the kidneys were changed into a thin hydronephrotic sac. However a few of the mice surviving beyond ten days did not show any signs of yeast infection at all.

Microscopical examination In the *acute type of disease* abscesses with fungi and inflammatory cells were found in the kidney, brain and heart. Both the yeast and pseudomycelial phase were seen in the lesions. Progressive infection was seen in the brain and heart in the animals dying in the acute stage and in the kidneys of animals in both types of infection. The other organs examined

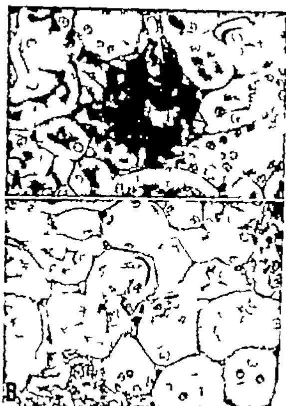


Fig 1 Section of mouse kidney 4 hours after intravenous injection of a sublethal dose of *Candida albicans*. A. Fungi in glomerular capillaries with mycelium extending into the capsular space. B. Fungi interstitially in renal cortex with mycelium penetrating into adjacent tubulus. Plastic embedded, PAS × 375

passed through a stage of limited infection which ultimately resolved. In the kidneys after 1 hour only very few yeast cells were observed in glomerular capillary loops and in cortical interstitial capillaries (Fig 1) At 8 hours pseudomycelial development resulted in penetration into the Bowman's space. After 24 hours a profuse invasive growth was seen interstitially issuing out of cortical capillaries. Inflammatory cells consisting of neutrophilic granulocytes and histiocytes were noticed in areas of damaged cortical tubuli. After 2–5 days the infection became more and more limited to large abscesses (Fig 2) and to the lumen of the tubuli. The fungi multiplied relatively unimpeded within the tubuli and subsequent pseudomycelial growth resulted in tubular perforation. This invasion of the interstitium was followed by a marked polymorphonuclear response and additional large abscesses developed. At the periphery of the abscesses there was a fairly wide zone containing numerous histiocytes, polymorphonuclear leukocytes and fungal elements, mainly pseudomycelium. After 7–8 days some epithelioid cell granulomas with occasional remnant fungal elements were observed in the cortex, while mostly pseudomycelium was seen in the collecting tubuli (Fig 3) Growth in this apparently protected site was not associated with the inflammatory response which was seen in the interstitium. After 8–9 days some scattered abscesses with fungi were noticed under the epithelium of the pelvis.

In the *chronic type of disease* no cortical abscesses, as seen in the acute type, were found, and only a few granulomas were left in the cortex. After 10 days fungi appeared in the deep calyces of the urinary pelvis. Be-



Fig 2 Section of mouse kidney 4 days after intravenous injection of *C albicans*. Abscesses in cortex with numerous fungi PAS × 20.



Fig 3 Section of mouse kidney 8 days after intravenous injection of *C albicans*. Fungi, mainly in the mycelial phase, in the collecting tubuli. Grocott × 380.

tween the 11th and 20th days partly necrotic tips of the pyramids were seen. There were adhering fungal masses (Fig 4 5 6) that increasingly filled the pelvis and protruded down into the upper part of the ureter The pelvic epithelium except on the papilla, showed no evidence of fungal invasion. In the papilla, at the border of the epithelial destruction, there were abundant inflammatory cells. Even the surface of the Candida bezoar in the pelvis was partly covered by inflammatory cells. The kidneys were unequally involved and occasionally only one kidney was involved. In one animal, after 25 days, a completely amputated tip of the pyramid surrounded by fungal masses and necrotic debris was seen in the renal pelvis. As the proliferating pseudomycelium and yeast cells caused a complete urinary blockage the hydronephrosis increased and after 30 days the kidney in some cases was changed into a hydronephrotic sac. In animals surviving this stadium with urinary obstruction there was often some resultant hydronephrosis. In the pelvis mainly fungal rests, necrotic debris and inflammatory cells were seen, but in some animals there were no bezoars or fungi at all.

Guinea pigs Three guinea pigs died due to the infection on the 3rd 5th and 6th days respectively These three animals were profoundly affected and lost, on an average, 200 grams in weight until their death. Up to the 15th day the guinea pigs lost weight, on an average, 40 grams. Thereafter they seemed to recover from the infection and gained weight. Macroscopical inspection of the viscera revealed changes only in the kidneys in the form of minute white abscess-like lesions up to the 12th day on the cortical surface. On section, these foci were usually found to be distributed throughout the cortex, occasionally extending into the medulla. Medullary lesions without cortical involvement were not

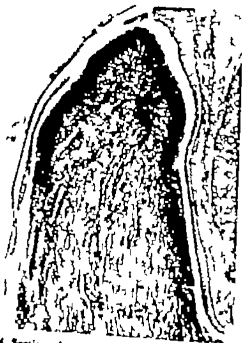


Fig 4 Section of mouse renal pelvis, 15 days after intravenous injection of *C albicans*. Accumulation of fungi adhering to the partly necrotic pyramid and partly filling the renal pelvis. Grocott × 40

Fig 5 Scanning electron micrograph of mouse renal pelvis, 20 days after intravenous injection of *C albicans* The pelvis is almost completely filled by a fungal bezoar adhering to the pyramid. SEM × 20



encountered. Neither papillary necrosis nor bezoars filling the renal pelves were observed.

Microscopical examination After 8 hours there were yeast cells in the glomerular capillaries and mycelial fragments were seen penetrating into the Bowman's space. After 1–2 days abscesses with collections of yeast cells and mycelium were seen mainly interstitially in the cortex. Later abscesses were observed even in the medulla. The pseudomycelial form dominated in the collecting tubuli however in some tubuli there were some neutrophil granulocytes and the yeast and mycelial forms were seen (Fig 7) Interstitial abscesses, even including the collecting tubuli, were formed by fungal invasion through the tubular epithelium. From the 6th day only inflammatory granulomas without Candida cells were observed, except for some vacuolated, fragmented fungal rests in the cortex. Fungi were growing out into the renal pelvis from the 9th day but no necrosis was observed in the papillary tips. There were no masses of fungi anchoring to the tips nor any bezoars in the renal pelvis After the 12th day only small scars lacking yeast elements were observed in the kidneys. In the heart and brain small abscesses with yeast cells and pseudomycelial filaments were found up to the 7th day

In the genitalia Candida cells surrounded by polymorphonuclear leukocytes were found in the ducts of the prostate of only one guinea pig killed after ten days. No fungal elements or inflammation were observed in the seminal vesicles or epididymes in any animal.

DISCUSSION

In recent years an increasing amount of attention has been paid to fungal infection in the urinary tract. One of the difficulties in relating results obtained through animal experiments to the human infectious state is that with animal experimental studies one usually has to work with considerably larger doses than those which initiate human infections

Candida albicans is a dimorphic mycete with a mycelium form, largely of pseudo-

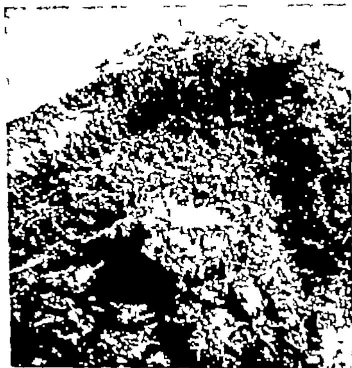


Fig 6 Scanning electron micrograph of tip of pyramid in mouse kidney 15 days after intravenous injection of a sublethal dose of *C albicans*. A. Numerous yeasts, mainly in the mycelial phase, are anchoring to the partly necrotic tip of the pyramid. SEM × 120

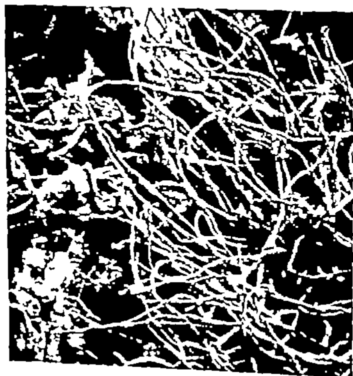


Fig 6 B. Fungi, mycelial phase, protruding out from distended collecting tubuli. SEM × 1200.

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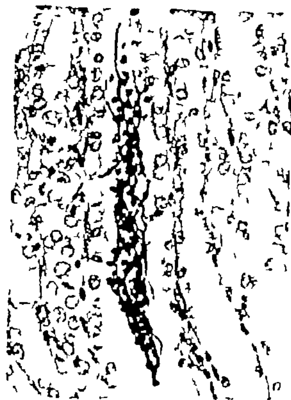


Fig 7 Section of guinea pig kidney 6 days after intravenous injection of *C albicans* Fungi, both yeast and mycelial phase, surrounded by polymorphonuclear leucocytes in a collecting tubulus. PAS × 380.

mycelial type, and a yeast like form, both occurring *in vitro* as well as in infections. The transition from the yeast form to the mycelial form can be stimulated by a number of different factors, one being treatment with broad-spectrum antibiotics, especially tetracyclines (*Simonetti & Strippoli* 1969) *Simonetti & Strippoli* (1973) showed in mice that the yeast form of *Candida albicans* in appropriate conditions, showed a significantly higher pathogenicity than the mycelial form after intraperitoneal and intravenous injection. However the mycelial form seemed to play an important role in the spreading of the infection.

In the present investigation in mice and guinea pigs it was possible to titrate a sublethal dose in order to get a more protracted infection with concomitant survival of most animals. The kidneys were the organ of maximum infection. In both animal species the kidney lesions in the acute stadium were of the same type as those seen in an acute haematogenous pyelonephritis in man. In the cortical Candida abscesses there was a proliferation of the inoculated yeast cells, as yeast cells as well as pseudomycelial elements were found. When the mice survived this stadium of infection they entered a chronic stadium with granulomas in the cortex and fungi in the collecting tubuli and particularly the renal pelves were filled with fungal masses. In some cases the pyramids had necrotic tips. Hydronephrosis with parenchymal atrophy was noticed. Both yeast and mycelial forms were attacked by the host defences and degraded. *Microscopical analysis indicated that* yeast cells haematogenously brought to the kidneys to some extent evaded the immune mechanism by invading in mycelial form intraluminally into the tubuli. In this protected environment the fungi rapidly and relatively undisturbed formed long mycelial elements. After rupture back into the interstitial tissues they were attacked primarily by polymorphonuclear leucocytes with subsequent abscess formation. In these abscesses a change into the yeast form was observed. This was earlier observed by *Louria et al.* (1960, 1963) who even observed that the inflammatory response in mice kidneys appeared four hours later than in other tissues. This later appearance in the kidneys was not seen in the present study in either animal species. *It is not clear* why the fungus within the renal tubular lumen should form long pseudomycelial elements, but *Blyth* (1964) suggests that the occurrence of yeast cells in areas containing leucocytic infiltrates indicates that the inflammatory cells were determining this phase in the dimorphy of the fungus. Recent *in vitro* studies (*Ozato & Uesaka* 1974) showed that *Candida albicans* phagocytized by rat phagocytes remained in the yeast form for about 2 hours *in a dormant state* but later some of them produced a pseudomycelium in the host cells, and ruptured the host cell membranes. Recently reported *in vitro* investiga-

tions clearly indicate the major responsibility of phagocytosis in inhibiting the development of Candida sepsis (*Stone et al.* 1974). In the absence of an inflammatory response, as in the uninfiltrated urinary pelves and collecting tubuli of the medulla, a pseudomycelium supervened.

In the present investigation, the transformation to the mycelial form was connected with progressive disease only in mice kidneys. The proliferation occurred mainly in the collecting tubuli and in the renal pelves. These experimental studies support the idea that the mycelial form is the invasive form in the kidneys. Similar observations have for example, been made in infants, where the change in predominance of yeast to mycelial forms in buccal smears is a reliable indication that oral thrush is imminent (*Taschdjian & Kozinn* 1957) and, in faeces, where the presence of predominantly the pseudomycelial form of *Candida albicans* has been suggested as an indication of overt intestinal candidiasis (*Rogers* 1957, *Kozinn & Taschdjian* 1962).

The inflammatory response in the mouse medulla was, as in our clinical case reports, moderate but in the guinea pigs abscesses sometimes developed. The microscopic picture with necrotic tips of the pyramids and renal pelves filled with fungi, as found in the chronic type of infection in mice but not in guinea pigs, was very similar to that seen in man (*Schönebeck & Winblad* 1971, *Schönebeck et al.* 1972). It is known that the hypertonicity in the medullary interstitium impairs leucocyte phagocytic function, rendering the medulla vulnerable to invasion by microorganisms (*Robbins* 1974). One could speculate that in mouse and man there is some factor in the medulla that stimulates the fungal growth or that the immunological defence mechanism is less developed. The ability of the fungal cells to propagate, enlarge in volume and occupy a progressively larger space mainly in the collecting tubuli in the medulla, and, possibly even through direct damage to the blood vessels, could cause a papillary necrosis in mice as has been observed in man. This picture was not observed in guinea pigs, where no fungi were seen in the kidneys after the 12th day.

Even in experimental kidney tuberculosis there are species differences between mice and guinea pigs. In guinea pigs it is impossible to induce a progressive kidney tuberculosis due to the kidney's resistance to tubercle bacilli (*Corper & Lurie* 1926, *Rich* 1944, *Birkhäuser* 1950) while a progressive infection leading to the ulcero-caseous stage is seen in mice (*Grumbach* 1958) corresponding to the picture seen in man. The pathogenesis of experimental renal candidiasis seems to resemble that of renal tuberculosis. *Medlar's* (1926) histological study of some 100,000 serial sections of kidneys from 30 persons who died from advanced pulmonary tuberculosis showed that the most common point of origin in the kidney was the capillary tuft of the glomerulus. Thereafter small lesions were found in the lumen of the tubuli and later down in the collecting tubuli in the pyramids. By means of animal experiments *Cosland* (1935) was able to confirm this route of spread in the kidney described by *Medlar*. In human cases renal candidiasis and renal tuberculosis may be confused both clinically and at necropsy (*Hurley & Winner* 1963) if the possibility of renal candidiasis is not born in mind.

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Acta path. microbiol. scand. Sect. A, 83: 415–424, 1975

EXPERIMENTAL MALE GENITAL TUBERCULOSIS—APPRAISAL OF PROTECTIVE VALUE OF BCG VACCINATION

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Winblad, B., Duchek, M. & Holm, S. Experimental male genital tuberculosis—appraisal of protective value of BCG vaccination. Acta path. microbiol. scand. Sect. A, 83: 415–424, 1975.

As part of an experimental study of the spread of urogenital tuberculosis, the development of tuberculous infection was compared in vaccinated and non-vaccinated guinea pigs after challenge with *Mycobacterium tuberculosis* strain $H_{37}R$ into the seminal vesicle. The animals were sacrificed 35 days after challenge. Morphological observations indicate that tuberculous infection in an immunized animal is retarded, but spreads by the same routes as in a non-immunized animal. On microbial enumeration significantly lower numbers of organisms were recovered from the inoculated seminal vesicle and regional iliac lymph nodes of the BCG-vaccinated guinea pigs.

Key words: Genital tuberculosis, male; experimental tuberculosis; BCG-vaccination; spread of tuberculosis; guinea pig.

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Vaccination with BCG induces a definite, although limited, level of antituberculous immunity in experimental animals and the guinea pig is an especially suitable experimental animal for this purpose facilitating the study of allergy and immunity in the same animal (e.g. *Jensen & Orskov* 1931, *Jensen & Bindslev* 1937, *Meyer* 1956, *Tolderlund et al.* 1967). Determination of the potency of a vaccine by means of the survival time of the experimental animal is the only non-arbitrary method. The majority of investigators have evaluated the effect of vaccination on the basis of macroscopic, sometimes completed with microscopic findings, combined with the results of culture from various organs, in vaccinated and non-vaccinated guinea pigs killed a certain time after challenge (e.g. *Fenner* 1951). However, the apparent potency of some vaccines is influenced by the animal model component of the test system (*Wiegeshaus et al.* 1971).

Different routes of administration of challenge have been employed in guinea pigs, mostly subcutaneous (e.g. *Sula* 1963), aerogenic (e.g. *Smith et al.* 1966) or intravenous (e.g. *Jespersen & Bentzon* 1967). Detailed knowledge of the natural course of the disease is essential for recognition of the true influence of vaccination. In our previous experimental investigations (*Duchek & Winblad* 1973a, b) the course of the tuberculous infection in the male genitalia after injection of tubercle bacilli, $H_{37}Rv$, in the seminal vesicle, epididymis and urinary bladder in

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ly bactericidal plastic material, well tolerated by tissues (Nobecutan® Bofors) was applied t the injection site and held in place for about a minute after the completion of the injection. The abdomen was losed in two layers.

The animals were killed 35 days after the challenge. However 3 animals in group III were kept alive for 5 months. The reason for this was the macroscopical observation that in animals autopsied after 35 days a spread of tuberculosis was noticed almost exclusively to the regional lymph nodes

Identification of Tuberculous Infection

The results were evaluated macroscopically microscopically and bacteriologically Autopsy was performed with sterilized instruments for each organ. The genital organs, kidneys, spleen, liver right lower lobe of the lung and iliac lymph nodes were divided into two parts one was used for culture while the other was fixed in formalin for histopathological examination. Sections were stained with hematoxylin-eosin and for fluorescence microscopy with auramine-rhodamine.

Enumeration of Living T bercle Bacilli in the Tissues

Weighed organ pieces were finely minced after addition of an equivalent amount of Sauton's medium From the homogenates 0.1 ml was spread over each of the surfaces of two Löwenstein-Jensen tubes and two tubes added with furan-2-carbonic acid hydrazide which in concentrations above 1 mcg/cc medium inhibits the growth of BCG but not of $H_{37}R$ As an extra control, cultured bacilli were niacin tested (*Jablin* 1960)

The tubes were incubated at 37 C, colonies were counted after 2 weeks and 6 weeks, and the results were used to calculate the number of cfu in the tissues.

RESULTS

All of the immunized guinea pigs (group I and III) reacted to intradermal tuberculin test with 2 TU PPD The mean size of the two diameters of tuberculin reactions in vaccinated guinea pigs was 9 (6–13) mm. The non-BCG-vaccinated animals (group II) showed no evidence of tuberculin sensitivity Isolated tubercle bacilli (group II and III) were niacin-positive indicating a human pathogenic strain.

Organs that were positive on culture almost always revealed mycobacteria upon fluorescence microscopy and tuberculous changes in light microscopy which is in agreement with our earlier observations (Winblad & Duchek, 1973)

Condition of Health and Demonstration of Tubercle Bacilli by Culture and Fluorescence Microscopy

In *group I* (animals 1–6) representing the control group of guinea pigs vaccinated with BCG without subsequent challenge, all animals gained weight, on an average 130 g during the observation period. No mycobacteria were cultivated from the organs. No culture was made from the site of vaccination but fluorescence microscopy revealed a few mycobacteria-like organisms.

TABLE 1 *Spread of T berculosis in the Male Uro-Genital System 35 Days after Inoculation of $H_{37}R$ into the Lumen of the Left Seminal Vesicle of Non-Vaccinated Guinea Pig (Group II)*

ANIMAL	LE	LSV	BP	RSV	RE	L	S	LU
7								
8	1	[illegible]	[illegible]		4	[illegible]	[illegible]	[illegible]
9		[illegible]	[illegible]			[illegible]		
10	[illegible]	[illegible]	[illegible]	3	[illegible]	[illegible]	[illegible]	[illegible]
11	[illegible]	[illegible]		42		[illegible]	[illegible]	
12	[illegible]	[illegible]	[illegible]			[illegible]	[illegible]	[illegible]
13	[illegible]	[illegible]				[illegible]	[illegible]	
14	[illegible]	[illegible]	[illegible]			[illegible]	[illegible]	
15		[illegible]	[illegible]			[illegible]	[illegible]	4
16		[illegible]				[illegible]	[illegible]	[illegible]

LE left epididymis.
LSV: left seminal vesicle.
BP bladder-prostate.
RSV: right seminal vesicle
RE right epididymis.
L: iliac lymph node.
S: spleen.
LU: lung.

The figures in the table indicate colony forming units (cf) per gram tissue. Dotted areas indicate identification of mycobacteria by fluorescence microscopy Animal 7 died in a disseminated tuberculosis on day 18.

In *group II* (animals 7–16) (see Table 1) representing the non-vaccinated guinea pigs inoculated in the left seminal vesicle with $H_{37}Rv$ one animal (7) died 18 days after the inoculation in a disseminated tuberculosis

with macroscopically visible lesions in the spleen and liver This animal had lost 180 g in weight. No culture was performed but fluorescence microscopy showed mycobac teria like organisms and by light microscopy tuberculous changes were observed in most organs. The other animals in the group increased on an average 40 g in weight during the 35 days of infection. As shown in Table 1 the highest bacterial counts (cfu) per gram tissue were obtained in the inoculated left seminal vesicle (LSV) and the corresponding regional iliac lymph node Tubercle bacilli were isolated from all animals at the site of inoculation and in the iliac lymph nodes.

In 4 of these animals high bacterial counts were obtained even in the homolateral epi didymis (animals 10 12, 13 14) and a few cfu in another two animals (8, 11)

There was spread to the prostate in most animals. In the majority of the animals tu bercle bacilli were recovered from spleen liver and lung with the highest counts mostly in the spleen. In the left kidney of two ani mals (8, 10) a few tubercle bacilli were found Culture on urine was positive in 3 animals (13 15 16) and on blood in one animal (12)

In *group III* (animals 17–26 and 27–29)



Fig 1 Mean counts of colony forming units (cfu) per gram tissue from the left seminal vesicle (LSV) and regional iliac lymph nodes (L) in guinea pigs inoculated with $H_{37}R$ into the lumen of LSV Compared re animals inoculated only with $H_{37}R$ (group II) and BCG-vaccinated animals inoculated with $H_{37}Rv$ (group III) Statistical analysis between the means of the counts show a signific t lower alue of cf in LSV and in L in the BCG- accinated animals compared to the non-vaccinated animals (Student's t-test $p<0.01$)

TABLE 2 *Spre d of Tuberculosis i the Male U o-Genital System in BCG-Vacc ated G nea Pigs after 35 Days I fection (Group III)*

Animal	LE	LSV	[illegible]	[illegible]	[illegible]	L	S	[illegible]
17		[illegible]				[illegible]		
[illegible]		[illegible]		[illegible]		[illegible]	[illegible]	
[illegible]		[illegible]	[illegible]			[illegible]		
[illegible]		[illegible]				[illegible]		
[illegible]		[illegible]				[illegible]		
[illegible]						[illegible]		
[illegible]		[illegible]				[illegible]	[illegible]	
[illegible]		[illegible]				[illegible]		
[illegible]		[illegible]				[illegible]		
[illegible]		[illegible]				[illegible]		
[illegible]								
[illegible]								
[illegible]								

Animals 7 29 were sacrificed 5 months after challenge with $H_{37}Rv$ The spread was evaluated only by means of fluorescence microscopy

(see Table 2) representing BCG-vaccinated animals inoculated with $H_{37}Rv$ no animal died and all gained weight, on an average 120 g during the 35 days after challenge. The infection was mainly limited to the inoculated seminal vesicle and the regional iliac lymph node The number of cfu in the seminal vesicles in these immunized guinea pigs was clearly lower than in the non vaccinated animals (group II) This trend was also noticed in the ilia lymph nodes (Fig 1) In 3 animals (18 21 23) bacilli were found in the spleen but not in the epididymis, liver and lung Cultivation on urine was positive in 2 animals (16 19) No BCG were recovered from the organs.

In the 3 guinea pigs (animal 27–29) that were left for 5 months after challenge re-



Fig. 2 Section of an iliac lymph node 35 days after injection of $H_{37}Rv$ into the lumen of the seminal vesicle of a non-vaccinated guinea pig (group II)

A. Part of the lymph node with confluent epithelioid cell granulomas. An epithelioid cell granuloma filling a dilated lymphatic vessel (arrow). Htx-eosin × 40.

B. Larger magnification of the granuloma which completely occludes the lymph vessel. Granulation tissue is seen protruding out into the surrounding tissue. Htx-eosin × 95

bercle bacilli in the tissues were sought only by means of fluorescence microscopy. These 3 animals all gained weight, on an average 270 g after challenge. In these animals tubercle bacilli were found not only in the seminal vesicle (animal 27, 28) and iliac lymph nodes but also in the left epididymis (animal 27, 28). Some single tubercle bacilli were found in granulomas of the lung in animal 27 and also in the spleen of the animals 27 and 28.

Morphological Findings

Gross lesions. In group I (only BCG-vaccinated) enlarged inguinal lymph nodes were observed in some of the animals. No other macroscopical changes were observed.

In all animals in group II (only inoculated with $H_{37}R$) the inoculated seminal vesicles were altered, occasionally appearing as stiff thick walled tubes. Streak formed changes were observed to the enlarged regional iliac lymph nodes and, in some animals, along the left ductus deferens to the left epididymis. Even paraaortic and renal lymph nodes were enlarged in most animals. In this group there was a wide-spread tuberculous dissemination. In all animals except one (animal 9) various degrees of enlargement of the spleen were seen and some, tuberculous changes with grey-white pin-head-sized flecks were noticed on the splenic surface. Similar flecks were found on the liver and lung in a few animals, corresponding rather well to the animals in which these organs were positive on culture.

In group III (BCG-vaccinated and inoculated with $H_{37}Rv$) the seminal vesicles showed minimal changes with slight wall stiffness, but in some animals they appeared quite normal. In all animals the regional iliac lymph nodes were enlarged. In animals 18 and 25 the spleen was enlarged and showed tuberculous changes. Otherwise no signs of

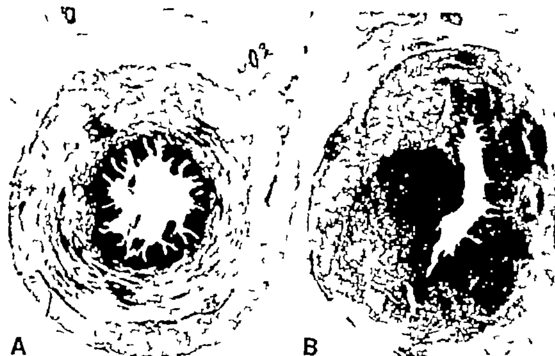


Fig 3 Section of seminal vesicle 35 days after injection of tubercle bacilli into the vesicle lumen.

A. BCG-vaccinated guinea pig (group III) Inflammation mainly in a slightly coarsed mucosa.
B. Non-vaccinated animal (group II) Tuberculous granulation tissue in the wall and scattered granulomas in the mesovesiculum. Htx-eosin × 25

miliary spread were observed. In animal 27 and less pronounced in animal 28 which were both sacrificed 5 months after the challenge tuberculous changes were observed in the left epididymis and along the left ductus deferens as grey white strings of beads as in group II.

icroscopic Observation

Group I Light microscopic examination of BCG-vaccinated animals did not reveal any specific changes in any of the organs except on the site of vaccination, where in some animals a tubercle with a central abscess and a fibrous capsule was seen.

Group II Thirty-five days after inoculation with $H_{37}Rv$ into the seminal vesicle the lumen was filled with an exudate containing polymorphonuclear leucocytes. Sometimes the lumen was almost completely obliterated, the mucosa ulcerated and the mucosal folds were coarsened, infiltrated with epithelioid cells, polymorphonuclear leucocytes and lymphocytes.

In some animals, almost throughout the whole wall, epithelioid cell granulomas with areas of necrosis were seen. Epithelioid cell granulomas were also seen around the vessels in the mesovesiculum.

The intensity of the inflammatory process in the iliac lymph nodes parallelled very closely that of the vesicle. The normal architecture of the iliac lymph nodes was almost completely replaced by epithelioid cell granulomas with some typical Langhans giant cells and central necrosis. In the lymphatic vessels epithelioid cell granulomas, that sometimes completely obturated the lymph vessel, were seen (Fig 2) In the prostate epithelioid cell granulomas were seen outside the wall of the inoculated seminal vesicle and interstitially surrounding the ductus deferens. In the epididymis tuberculous changes were seen in the interstitial tissue No inflammatory changes were noticed in the lumen of the ductus



Fig. 4. Section of funiculus spermaticus adjacent to epididymis in a BCG-vaccinated animal killed 5 months after injection of $H_{37}Rv$ into the seminal vesicle (group III). In the tissue outside the intact ductus deferens a tuberculous granuloma is seen. Hta-eosin × 95



Fig. 5. Section of epididymis and testis in the same animal as in Fig. 4. Tuberculous granulation tissue with areas of necrosis partly invading interstitially in the epididymis. Testis (right) uninvolved. Hta-eosin × 23

deferens. No tuberculous changes were ever seen in the testis.

In the spleen the lymphatic nodules were partly replaced by epithelioid cell granulomas. In some animals small necrotic lesions and abscesses were distributed throughout the parenchyma.

In the liver of some animals there were, mainly in the periportal area, small granulomas which contained lymphocytes, neutrophiles and epithelioid cells. In some of them typical Langhans giant cells were seen.

In the lungs typical tubercles with epithelioid structure and Langhans giant cells were only found in 2 animals (8, 10). The lesions were of a proliferative type, no necrotic-exudative lesions were found. The kidneys showed no specific changes.

Group III. The most striking effect of BCG vaccination was noticed in the inoculated seminal vesicles of animals 17–26 where only slight changes compared to the non-vaccinated animals (group II) were seen (Fig. 5). The changes consisted of inflammation in a slightly coarsened mucosa and in 4 animals (17, 18, 19, 26) a few epithelioid cell granulomas were seen in the wall. No necrosis was found in the vesicles. The tuberculous tissue reaction did, however, not differ qualitatively from what was found in the non-immunized animals.

In the iliac lymph nodes the difference was not so striking. Usually a smaller part of the lymph gland was replaced by epithelioid cell granulomas, and in 5 animals (17, 18, 22, 23, 26) small necrotic areas were observed.

In the spleen confluent epithelioid cell granulomas were observed in animals 18 and 23. In animal 23 small necrotic areas were seen. In animal 19 an abscess was observed interstitially in the prostate.

In animals 27–28 (killed 5 months after challenge), however, epithelioid cell granulomas were observed in the funiculus sper-

matilcus outside an intact ductus deferens (Fig 4) and in the epididymis (Fig 5) Tubercles were found in the lung of animal 27

DISCUSSION

The purpose of these experiments was to investigate if the immunity induced by BCG-vaccination in guinea pigs prevents the progress of tuberculosis when tubercle bacilli are introduced into the genitalia. There is definite evidence that the immunity that develops after vaccination is manifested by retardation of multiplication of the tubercle bacilli introduced into the host by other routes of administration in experimental animals (*Levy et al* 1961) The immunogenic effect can be determined by comparing the course of tuberculosis in a non-vaccinated group of animals with the infection in a group of vaccinated ones. As a further control group BCG-vaccinated, unchallenged animals should be used. The latter group is motivated as it is not possible to distinguish virulent bacteria and BCG by means of fluorescence microscopy or by colony morphology (*Jespersen* 1971) This differential diagnosis can be performed however using information concerning special biochemical characteristics for the two isolated mycobacterial strains (*Juhlin* 1960)

The acquired resistance evoked by BCG vaccination is relative and can only be demonstrated under certain experimental conditions. *Willis et al.* (1960) found after extensive studies in guinea pigs that a large challenge dose tends to overwhelm immunity They stated that relatively small challenge doses (30 000 to 60 000 cfu) were most suitable to demonstrate if vaccination afforded protection against the infection. There are difficulties in obtaining a standardized dose as the number of cfu is not known at the time of the challenge. Our challenge dose turned out to be about 40,000 cfu. This dose is about tenfold higher than in our earlier investigations (*Duchek* & *Winblad* 1973b) The dose used in the present investigation is probably larger than the dose usually encountered in nature. The cfu in the inoculum and the homogenized organs is certainly less than the real number of viable bacteria. This is due to the fact that although thorough dispersion there will always be clumps with many mycobacteria in the inoculum. Further it is difficult to attain a complete mechanical communition of the organs (*Jespersen* 1971)

The survival time after challenge has often been used as an objective measure of acquired resistance (*Tolderlund et al.* 1967 *Jespersen* & *Bentzon* 1967) In the present investigation all the animals were killed five weeks after inoculation. This time was chosen as the vaccination allergy reaches its maximum within 1½ months and remains at this level for about one month (*Tolderlund et al.* 1960) Macroscopical and microscopical examinations provide qualitative information of the spread and the type of lesions. Slight discrepancies were noted between the two methods used for demonstration of tubercle bacilli, cultivation and fluorescence microscopy. This can be explained by the difficulties encountered in sectioning representative pieces for both histological methods and cultivation. Another explanation could be that the tubercle bacilli were already dead or were so few in number that they were not represented in all sections.

The protective effect of BCG-vaccination was obvious, as in the vaccinated animals in contrast to the non vaccinated ones, the infection was mainly limited to the inoculated seminal vesicle and the regional iliac lymph nodes and the number of cfu was significantly lower. The more wide-spread dissemination in the non-vaccinated animals than that seen earlier (*Duchek* & *Winblad* 1973b) is probably explained by the higher dose used in the present study. Because of their use, bacteria readily enter the lymphatics but not the blood capillaries. *Barnes* & *Trueta* (1941) clearly showed this in rabbits. *Bergman* (1961) showed that fungi (*Cryptococcus neoformans*) injected subcutaneously into the hind foot of mice after obstruction of blood flow travelled via the lymphatics before entering the blood stream from which they could

be cultured within 1 hour after the injection. Ligation of the femoral blood vessels had no effect on the transport of the fungi, while ligation of the lymphatics hindered the transport. There was no difference in the dispersion of "living" and killed fungi. This finding is in agreement with that of *Boquet et al* (1931) after subcutaneous injection of living or killed tubercle bacilli in guinea pigs.

In the present investigation it seems that in the vaccinated animals the infection is retarded at the site of inoculation and in the regional lymph nodes through a defence mechanism on an immunological basis. This is in striking contrast to the findings in the non-immunized animals where the dose overwhelmed the immunologically unspecific defence mechanisms and the bacilli passed through the lymph nodes and entered the blood stream and provoked a wider dissemination. However a limited degree of multiplication proceeded also in the vaccinated animals, locally on the site of inoculation and in the regional lymph nodes. At the time chosen for autopsy there was no spread to the scrotal genitalia but indications of a progressing infection were found in the 3 vaccinated animals that were kept alive for 3 months after the challenge. In 2 of them spread was noticed from the inoculated left seminal vesicle along the ductus deferens to the homolateral epididymis. In sections of the funiculus spermaticus tuberculous changes were observed in and around the lymph vessels, but no changes were observed in the ductus deferens. This mode of spread is in agreement with our earlier studies (*Dukek & Winblad* 1973a, b). The number of animals is, however, too small to enable definite conclusions to be drawn. Probably a longer time of infection and/or a higher challenge dose in the vaccinated animals would have resulted in a wider extent of involvement of the lymphatic system.

These macroscopical and histopathological observations together with fluorescence microscopy and quantitative cultivations show that BCG-vaccination has a suppressive effect on genital tuberculosis in guinea pigs. Vaccination does not prevent the establishment of local infection, but merely retards its course and delays a miliary spread. Similar observations have been made in pulmonary tuberculosis in man (*Wallgren* 1948, *Lindgren* 1965).

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MALE GENITAL TUBERCULOSIS— THE POSSIBILITY OF LYMPHATIC SPREAD

A Case Report

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Winblad, B. Male genital tuberculosis—the possibility of lymphatic spread. A case report. Acta path. microbiol. scand. Sect. A, 83 425-428, 1975

A 32-year old man with recurrent, persistent tuberculosis developed a tuberculous epididymitis. No inflammatory changes were found in the ductus deferens. It is likely that spread of infection from the prostate to the epididymis occurred via the lymphatics in the *funiculus spermaticus*.

Key words Genital tuberculosis, male lymphatic spread.

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There is no unanimity of opinion as to how tuberculous infection reaches and spreads within the male genitalia. Nowadays, however most authors are of the opinion that genital tuberculosis arises by urinary spread to the pelvic genital organs (*Joller* 1949 *Mazurek* 1963, *Kuhn* & *Unger* 1966 *Albers* 1970) and that there is an intracanalicular spread via the ductus deferens to the epididymis (*Borthwick* 1946 *Joller* 1949 *Medlar et al* 1949) Blood-borne infection to the testis and epididymis has been observed in a few cases (*Borthwick* 1946) The possibility of lymphatic spread from the pelvic genital organs to the epididymis has been shown experimentally in the guinea pig after resection and ligature of the sperm duct (*Du kek* & *Winblad* 1973) As the following case illustrates, lymphatic spread to the epididymis is also possible in man.

CASE REPORT

The patient, a 32-year old male farmer married, with two children born in 1946 and 1950. Until 1942, at age 20 yrs, he had been in good health. The first sign of illness was in the spring 1942, when he had a bad cold and afterwards complained of fatigue In November 1942, chest x-ray showed left-sided adhesions of the pleura and dense areas in the apical region of both lungs. In 1944 chest x-ray control showed progress of the apical changes, and they were diagnosed as chronic, productive apical tuberculosis Cultures and guinea pig tests of the stomach fluid were negative The patient was released after 6 months sanatorium care and was checked regularly thereafter

In april 1947 the patient developed a left-sided epididymitis. Upon examination the left scrotal half was swollen, the skin reddened but moveable above the infiltrate. The left epididymis was firm and infiltrated with a hazelnut-sized tumour in the cauda. There was also infiltration a little further up the epididymis. The ductus deferens was not particularly thickened, but the funiculus spermaticus, on the whole, palpated somewhat firmer than normal. The right testis was somewhat atrophied (had been so since a hernia operation at age 1 yr) right epididymis normal. Per rectum the left vesicula seminalis was palpated as swollen and relatively firm and infiltrated The prostate was some-

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RELATIONSHIP BETWEEN HEART WEIGHT AND THE CROSS SECTIONAL AREA OF THE CORONARY OSTIA

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Paulsen, S., Vetner M. & Hagerup, L. M. Relationship between heart weight and the cross sectional area of the coronary ostia. Acta path. microbiol. scand. Sect. A, 83: 429–432, 1975

Sex variation in the area of the coronary ostia was studied in hearts with a normal weight (heart weight <400 g) and hypertrophic hearts (heart weight ≥400 g). Also studied was a possible sex difference in the correlation between heart weight and the area of the coronary ostia. The study was performed on 379 post mortem hearts. In the group of hearts with a normal weight the area of the left coronary ostium in both sexes was greater than the right but the difference was statistically insignificant. There was no sex difference in the area of the left coronary ostium, whereas the area of the right coronary ostium was statistically significantly less in women. In the group of hypertrophic hearts the left coronary ostium increased in area with increasing heart weight. The difference in the area of the left coronary ostium between hearts with normal weight and hypertrophic hearts was statistically significant in men, but not in women. The right coronary ostium showed minimal increase in size with increasing heart weight and the difference in both sexes between the two groups was statistically insignificant. In the group of hypertrophic hearts the heart weight was best correlated to the area of the left coronary ostium in both sexes, but the values did not reach statistical significance. On the basis of this study the area of the right coronary ostium appears to be a bottleneck with regard to an adequate blood supply to a hypertrophic myocardium.

Key words: Heart weight; coronary ostia area.

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The ostia of the coronary arteries play a significant role in the provision of an adequate blood supply to the myocardium. We have been able to find only a single study on the size of the coronary ostia (Vogelberg, 1957) and on search of the literature from 1965 to April 1974 using electronic data registration (Medlars and Medline, Karolinska Institutet, Stockholm) no such studies were found.

The purpose of the present study was to investigate whether a sex related difference exists between the area of the coronary ostia in hearts of normal weight and hypertrophic hearts and whether there was a sex related correlation between heart weight and the area of the coronary ostia.

MATERIALS AND METHODS

Fourhundred unfixed hearts from 400 consecutive autopsies during a nine month period were studied. Because of anatomic variations and stenosing atherosclerosis of the ostia, twenty-one hearts were

discarded Several rightsided coronary ostia are more common as an anatomic variant than are left-sided ostia which are rather rare In our study one case was found with a single coronary artery and one case with two ostia on the left side Both of these hearts were discarded. On the right side we only measured the main ostium since any other ostia represented the origin of rather small arterial branches supplying a limited area of the anterior wall of the right ventricle

The study material wa divided into two groups. Group I consisted of hearts with a normal weight (heart weight <400 g) whereas group II consisted of hypertrophic hearts (heart weight $\geqq 400$ g) Measurement of the area of the coronary ostia wa undertaken on open cut ostia with the help of a compass. Measurement was an approximation of the circumference and was used in calculating the area. All measurements were undertaken by the same person (M.V)

The statistical calculation were performed with the help of Kruskal Walli test and Spearmans rank correlation test. The ni eau for statistical significance was $p \leqq 0.05$

RFSULTS

Sex, age and heart weight data for the entire study material are given in Table 1

Group I

Sex distribution average age mean heart weight and area of the left coronary ostium, right coronary ostium and the sum of both coronary ostia in hearts of normal weight are given in Table 2. There were 79 men with an average age of 62 years (range 11–85) and 92 women with an average age of 65 years (range 14 90) Heart weight was significantly greater among men ($p<0.05$). There was a slight difference in the area of the left and right coronary ostium in men whereas the corresponding difference in women was greater Both differences were, however statistically insignificant. There was no sex related difference in the area of the left coronary ostium whereas the sex difference for the right coronary ostium was marked and statistically significant ($p<0.01$) Calculation of the area of the left and right coronary ostium in men and women in the various age groups showed that there was no statistically significant difference in the area of the ostia in relationship to age.

An analysis of the correlation between *heart weight and area for the right*, left and sum of both coronary ostia in men and women demonstrated no correlation in men. In the corresponding analysis in women a statistically significant correlation was found (heart weight/left coronary ostium $p<0.05$, heart weight/right coronary ostium $p<0.01$ and heart weight/both coronary ostia $p<0.01$)

Group II

Sex distribution, average age, mean heart weight and area of the left coronary ostium, right coronary ostium and sum of both coronary ostia are given in Table 3. There were 146 men with an average age of 68 years (range 36–9?) and 62 women with an aver

TABLE 1 *Age and Sex Distribution of the Study Material*

Age group	Men		Total	Women		Total
	Heart weight			Heart weight		
	<400 gm	$\geqq 400$ gm		<400 gm	$\geqq 400$ gm	
40	7	1	8	4	0	4
40–49	10	4	14	9	2	11
50–59	5	23	28	16	2	18
60–69	29	51	80	23	20	43
70–79	21	44	65	26	29	55
80	7	23	30	14	9	23
Total	79	146	225	92	62	154

TABLE 2. *Sex Distribution, Average Age, Mean Heart Weight and Area of the Left Coronary Ostium, Right Coronary Ostium and Sum of Both Coronary Ostia in Hearts of Normal Weight*

Sex	Age (years) mean	No. of hearts	Heart weight (gm) mean ± SD	Left coron. ost. (sqmm) mean ± SD	Right coron. ost. (sqmm) mean ± SD	Sum left and right coron. ost. (sqmm) mean ± SD
M	62	79	319 ± 47	7.8 ± 3	7.2 ± 2.2	15.7 ± 3.6
F	65	92	297 ± 65	7.8 ± 2.9	6.1 ± 2.2	13.9 ± 3.9
Total		171	307 ± 58	7.8 ± 2.9	6.6 ± 2.3	14.4 ± 3.8

TABLE 3. *Sex Distribution, Average Age, Mean Heart Weight and Area of the Left, Right Coronary Ostium and the Sum of both Coronary Ostia in Hypertrophic Hearts*

Sex	Age (years) mean	No. of hearts	Heart weight (gm) mean ± SD	Left coron. ost. (sqmm) mean ± SD	Right coron. ost. (sqmm) mean ± SD	Sum left and right coron. ost. (sqmm) mean ± SD
M	68	146	545 ± 103	9.9 ± 4.5	7.6 ± 3.5	17.4 ± 6.2
F	72	62	486 ± 91	8.9 ± 3.8	6.5 ± 2.7	15.4 ± 5.0
Total	69	208	527 ± 104	9.6 ± 4.3	7.3 ± 3.3	16.8 ± 5.9

age age of 72 years (range 41–92). Heart weight among men was significantly greater ($p < 0.01$). There was a greater difference in the area for the right and left coronary ostium in men than in group I and the difference was significant ($p < 0.01$). In women the difference was also greater than in group I but statistically insignificant. A comparison between sex differences in area of the left and right coronary ostium and the sum of both coronary ostia revealed that there was no statistically significant sex difference in the left and right coronary ostium whereas sex difference was significant regarding the sum of both coronary ostia ($p < 0.05$).

A comparison between the area of the left and right coronary ostium and the sum of both coronary ostia in men and women in group I and group II showed that all values were higher in group II than in group I. The difference was statistically significant in men for the left coronary ostium ($p < 0.01$) and the sum of both coronary ostia ($p < 0.01$) and in women for the sum of both coronary ostia ($p < 0.05$).

An analysis of the relationship between heart weight and area for the left and right coronary ostium and the sum of both coronary ostia in both sexes, showed the best correlation between heart weight and area of the left coronary ostium, but the correlation was not statistically significant (female $p = 0.1$, male $0.1 > p > 0.05$). The remaining calculations showed completely insignificant values.

DISCUSSION

The border between normal heart weight and cardiac hypertrophy is indistinct and an arbitrary limit of 400 g was chosen even though this obviously represents a rather rough classification. A more exact separation would probably be obtainable if the myocardium of the left and right ventricles were weighed separately after removal of the atria, valvular apparatus, coronary arteries and the subepicardial fat (Lamb 1973).

Our method of measurement was not numerically exact but since the purpose of the

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present study was to evaluate differences and correlations this inaccuracy was accepted. *Fogelberg* (1957) found greater absolute values because another method of measurement was used. In *Fogelberg's* study measurement of the coronary ostia was undertaken in 225 normal formaline fixed hearts (heart weight ≦350 g) and 250 hypertrophic formaline fixed hearts (heart weight >400 g). Results were not statistically evaluated. *Fogelberg* measured the coronary ostia in normal hearts in age groups from the newborn to senility. In newborns he demonstrated that the area of the left and right coronary ostium was the same. Approximately 4 weeks after birth and up to the age of 20 the area for both coronary ostia revealed a regular steady increase. In the age group between 20 and 30 years the area of the left coronary *ostium increased more than that of the right* whereas after the age of 30 the increase in area stopped for both coronary ostia. The former measurements cannot be compared with ours since our study material contained only 3 patients less than 20 years of age. On the other hand we can confirm the findings that there is no increase in the area of the coronary ostia after the age of 30 since we were not able to demonstrate any statistically significant difference in the various age groups in both sexes in group I. On the other hand we found in contrast to *Fogelberg*, that the area of the right coronary ostium was less in women than in men and that this difference was statistically significant.

Fogelberg undertook a subgrouping of hypertrophic hearts for each 100 g but without sex differentiation. He found that the left coronary ostium increased in area for hearts up to 500 g. After this weight class the area of the left coronary ostium remained stationary whereas there were less differences with regard to the right coronary ostium compared with values in normals. Even though we undertook a separation on the basis of sex there appears to be a certain harmony between the results of *Fogelberg* and ours.

In our study the difference in men in the area of the left coronary ostium between hearts of normal weight and hypertrophic hearts was statistically significant, whereas the difference showed the same tendency in women but was statistically insignificant. Corresponding to *Fogelberg* we found that the difference in the area of the right coronary ostium between the two groups was minimal and statistically insignificant and this was the case for both sexes. The heart weight was best correlated to the area of the left coronary ostium in the hypertrophic hearts in both sexes, but the values did not reach statistically significance.

The blood supply to the myocardium must pass through the coronary ostia. On the basis of our study we suggest, that the left coronary ostium increases with increasing heart weight whereas the area of the right coronary ostium is almost stationary and unaffected by increasing heart weight. This appears to give the right coronary ostium a commanding influence on the blood supply to the myocardium. This will be of critical importance in cases of cardiac hypertrophy and pronounced dominance of the right coronary artery. In such cases the right coronary artery is responsible for greater portion of the blood supply to the myocardium of the left ventricle.

Statistical calculations were carried out at the Department of Medical Data Processing, Gentofte Hospital, 2820 Gentofte, DK.

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Acta path. microbiol. scand. Sect. A, 83 433–442, 1975

FINE STRUCTURE OF GRANULOCYTES WITH CYTOPLASMIC INCLUSIONS IN PLEURAL EFFUSIONS FROM PATIENTS WITH RHEUMATOID PLEURITIS, TUBERCULOUS PLEURITIS AND PLEURAL CARCINOMATOSIS

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Nielsen, M. H., Faurschou, P. & Faarup, P. Fine structure of granulocytes with cytoplasmic inclusions in pleural effusions from patients with rheumatoid pleuritis, tuberculous pleuritis and pleural carcinomatosis. Acta path. microbiol. scand. Sect. A, 83 433–442, 1975

The fine structure of neutrophil granulocytes from pleural fluid obtained from patients with rheumatoid or tuberculous pleuritis or epidermoid carcinoma of the lung was identical ir respectiv of the primary disease of these patients. Homogenously stained lipid-like inclusions were present in the cytoplasm of the neutrophil granulocytes. They seemed to be formed *de novo* as they lacked a distinct limiting unit membrane. The fine structure of the lipid-like inclusions gav no evidence to support the presence of immune complexes, these were probably located in the quantitative much fewer phagosomes of the granulocytes.

Key words: Neutrophil granulocyte with cytoplasmic inclusions, pleural effusions, rheumatoid pleuritis, tuberculous pleuritis, pleural carcinomatosis.

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Neutrophil granulocytes with cytoplasmic inclusions—termed ragocytes or RA-cells—were previously believed to occur only in serous effusions from patients with rheumatoid arthritis or rheumatoid pleuritis (1, 3, 5, 11, 16). Recently however similar cells were identified in pleural fluid from patients with tuberculous pleuritis of the lung (8), as well as in patients having malignant pleural affections (9).

Previously the fine structure of granulocytes with cytoplasmic inclusions has been studied only in cells from the rheumatoid affections (11, 21). It has been the object of the present investigation to compare the ultrastructure of such pleural cells in patients suffering from rheumatoid pleuritis, tuberculous pleuritis as well as pleural carcinomatosis caused by lung carcinoma.

TABLE 1 *Total Leucocyte Counts and Differential Distribution of Neutrophil Granulocytes and Mononuclear Cells in the Pleural Fluid*

Case No.	Sex	Age	Diagnosis	Leucocytes per ml	Per cent neutrophil granulocytes	Per cent mono-nuclear cells
1	♂	60	Rheumatoid pleuritis	3.450	60	40
2	♂	47	Tuberculous pleuritis	2.256	1	99
3	♂	40	"	2.300	22	78
4	♀	75		10.827	84	16
5	♀	75		13.800	78	22
6	♂	73	Epidermoid carcinoma of the lung	2.000	18	72
7	♂	61		160.000	95	5

MATERIAL AND METHODS

One patient with rheumatoid pleuritis, four patients with tuberculous pleuritis, and two patients with carcinomatosis of the pleura secondary to bronchogenic carcinoma were included. Thoracocentesis and thoracoscopy was performed in all patients, and pleural biopsies (visceral and parietal) and pleural fluid were obtained for histological and cytological examination for neutrophilic granulocytes with cytoplasmic inclusions (8) and for electron microscopy

Clinical Data

The patient with rheumatoid pleuritis was a middle-aged man (Table 1) with unilateral pleurisy He fulfilled the criteria for rheumatoid arthritis set up by the American Rheumatism Association (19) His pleural fluid had a low glucose content (10 mg/100 ml) and a high lactate dehydrogenase activity (2600 unit/liter) Repeated examination for t mor cells and tubercle bacilli in the pleural fluid were negative. On thoracoscopy the parietal pleura wa found moderately inflamed with vesicles or nodules about 0.5 mm in diameter scattered all over the surface These findings are considered to be diagnostic of rheumatoid pleuritis (9)

In the four patients with tuberculous pleuritis *Mycobacterium tuberculosis* was identified in the pleural fluid.

In the two patients with pleural carcinomatosis, the primary tumors were epidermoid bronchogenic carcinoma and bronchogenic combined epidermoid and adenocarcinoma This was confirmed at autopsy

Preparation for Electron Microscopy

Pleural biopsies were immediately placed in Karn sky s f xative (15) diluted 1:1 with distilled water divided into blocks (1 × 2 × 2 mm) with razor blades and fixed for 3 hours.

Pleural fl id was mixed with an equal volume of 3 per cent gl taraldehyde in 0.2 M cacodylate buffer pH 7 and fixed for 20 minutes It was then centrifuged at 3000 × *g* for 10 minutes, and the pellet gently divided in blocks (2 × 2 × 2 mm), which were fixed for 1 hour in 1.5 per cent glutaraldehyd in 0 1 M cacodylate buffer pH 7.2 Tissue blocks and small blocks of the pellet were washed at 4 C i 0 2 M cacodylate buffer pH 7.2 with 0.2 M sucrose added for 24 to 72 hrs. Post-fixation for 2 hrs was performed with 1 per cent osmium tetroxide in cacodylate buffer pH 7.2. The cell were then washed in cacodylate buffer dehydrated in graded ethanol followed by propylene oxide, and finally embedded in Vestopal W Appropriat areas for electron microscopy were chosen by light microscopic examination of 1 μm sections stained with toludin blue. Ultrathin sections were made on an LKB Ultrotome. They were stained with uranyl acetate (12) and lead citrate (18) and examined in Siemens Elmiskop 1A.

RESULTS

Light Micros opy of Pleural Effusions

The total number of leucocytes in the pleural fluid, and the proportion of neutrophil granulocytes and mononuclear cells varied considerably from one patient to another (Table 1) In all patients, however

granulocytes with cytoplasmic inclusions were identified in vitally stained specimens. In most fluids the majority of mononuclear cells were macrophages, whereas lymphocytes comprised only a few per cent however in one patient with carcinoma of the pleura (case no. 6) the majority of mononuclear cell was tumor cells.

From 5 to 30 per cent of the morphologically intact neutrophils displayed large spherical cytoplasmic inclusions which could be stained with lipid stains such as Sudan Black or Scharlach R, (L, Fig. 1) Lipid granules were also present in many macrophages, and in a few of the lymphocytes.

Cell debris, lipid granules and precipitates of fine granular or amorphous material and fibrin were present—to a varying extent—in all pleural fluid examined.

Neutrophil Granulocytes

Electron microscopy of pleural effusions The fine structure of the neutrophil granulocytes varied from one cell to another. This was true not only of the number and size of lipid-like inclusions and phagosomes, but also of the content of specific neutrophilic granules, as some cells were almost completely degranulated. However there were no marked differences between the morphology of neutrophils from patients with rheumatoid pleuritis, tuberculous pleuritis and carcinomatosis of the pleura.

Cytoplasmic inclusions On electron microscopy spherical cytoplasmic inclusions of lipid-like material (L, Figs. 2, 3 4 6 7) were found in about half of the neutrophils. They measured about 1 µm in diameter and consisted mainly of amorphous material which was homogenously stained and of a moderate electron-density. The inclusions often displayed spherical electron translucent areas—apparently formed by extraction—toward the center (V Fig 2) The most peripheral layer of the lipid like inclusions had increased electron-density but showed normally no distinct limiting membranes (Fig. 3 P Figs. 6 7) Lipid like inclusions which at least in part



Fig. 1 Smear of sediment from pleural fluid stained with Scharlach R. Neutrophil granulocyte with lipid granules (L) Extracellular lipid granules in the pleural fluid are marked with an arrow Magn. × 1,680.

were limited by a membrane were few (L, Fig. 4)

Phagosomes. Membrane-limited phagosomes (PV Figs. 2, 4 5) were less frequently observed than lipid-like inclusions. They were

Fig 2 Cells from pleural effusion of case 6 Most cells are neutrophils. Cell debris (D) a single erythrocyte (E) and an extracellular lipid droplet (EL) are also present. The neutrophils display lipid-like inclusions (L) with a varying number of clear vacuoles (V) A probable phagosome is marked PV Magn. × 6,000

Fig 3 Neutrophil granulocyte from the pleural effusion of case 3 A lipid-like inclusion with dense periphery is marked L. Small and medium sized specific granules are marked SG N denotes the nucleus. Magn. × 17,000.

Fig 4 Part of a neutrophil granulocyte from the pleural exudate of case 3. A probable membrane limited lipid-like inclusion (L) and a phagosome (PV) are visible N denotes the nucleus. Magn. × 30,000


D
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V
L
D
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E
2

SG

L

SG

3

0,5 µ

PV

4



Fig 5 Neutrophil granulocyte from the pleural effusion of case 1 The phagosomes (PV) contain flocculent material of moderate electron-density membranes (M) and cellular cell debris (D) The specific neutrophilic granules (SG) are not intensely stained. N denotes the nucleus. Magn. × 21,000.

of approximately the same size and enclosed a varying amount of electron-dense material, some of which was identified as being of extracellular origin, such as cell debris (D Fig 5) or fibrin precipitates, but some was less well defined flocculate material.

Neutrophilic granules The cells usually had two distinct types of neutrophilic granules (SG Figs 3 5 6) One appeared as a large granule with a light matrix and one as a small granule with an electron dense matrix. A very high proportion of neutrophils, however displayed granules all of which had low electron density (SG Fig 3) probably due to

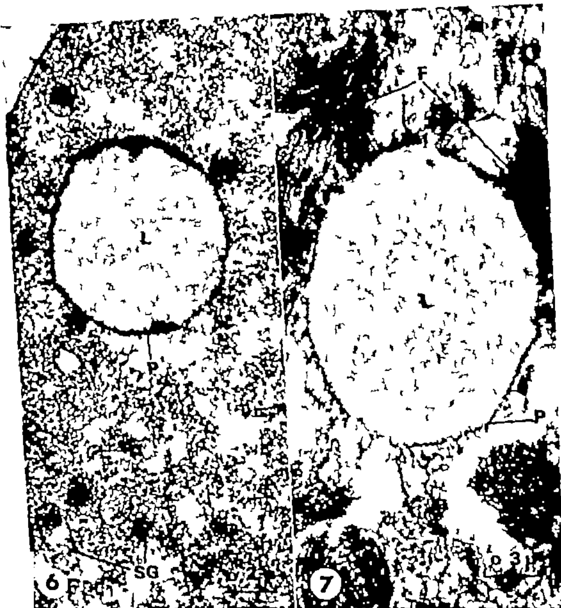


Fig 6 Part of a neutrophil granulocyte from case 3. Lipid-like inclusion (L) with a homogenously stained core of moderate electron density and dense peripheral part (P) No distinct limiting membrane is visible around the lipid-like inclusion, whereas the small specific granules (SG) clearly are membrane bound. Magn × 56,000

Fig 7 Free lipid-like granule (L) in the fibrous part of parietal pleura from case 3 The core is unevenly stained The dense peripheral part (P) displays no ordinary membrane. Fibrin precipitates are marked F Magn × 47,000

extraction of matrix. In these cells the different granules could be identified only because of their different size and shape

The large granules were usually ellipsoid but sometimes spherical, and measured approximately 0.2 × 0.3 μm in diameter The small granules were usually spherical, but rodshaped or dumbell-shaped granules were

frequently encountered. Their small diameters were less than 0 1 μm. *The granules were all* limited by an ordinary triple-layered membrane (Fig 6) None of the granules contain *elelectron-dense inclusions or crystalloids.* No large "azurophilic granules (20) were observed.

The neutrophil granulocytes contained very few mitochondria and almost no granular endoplasmic reticulum.

Lymphocytes macrophages free tumor cells Lipid inclusions as described above were occasionally also present in these cells.

Electron Microscopy of Pleural Biopsies

Neutrophil granulocytes and free spherical lipid like droplets without limiting membrane (L, Fig 7) were observed in the pleural biopsies from two patients only (case nos. 5 and 7) They were located in the fibrinogen-containing layer of non-cellular material which formed the luminal part of the biopsies from one patient with tuberculosis (case no. 5) and one patient with carcinoma of the pleura (case no. 7) The neutrophils of the *pleural biopsies contained fewer lipid* inclusions and fewer large phagosomes than the neutrophils of the pleural fluid.

The deep cell-containing part of the pleural biopsies—whether lung tissue dense connective tissue granulation tissue or tumor tissue —*contained no neutrophils.*

DISCUSSION

In the present work the ultrastructure of granulocytes with cytoplasmic inclusions in the pleural fluid from patients with rheumatoid and tuberculous pleuritis, and pleural carcinomatosis were compared.

In view of the fact that the principal disease behind the pleural affections observed was quite different in the three categories of patients, it was surprising to find that the *major* cellular components of the pleural effusion were of the same fine structure, and in particular that the contents of the cytoplasmic inclusions of the neutrophils showed no ultramicroscopic differences. This may *well suggest* that in the three groups of patients examined, principally the same pathogenic mechanism is active in the development of pleural neutrophil granulocytes with cytoplasmic inclusions—a hypothesis which finds support in the parallel biochemical data obtained (10)

The most conspicuous structures in the neutrophils were the large lipid-like cytoplasmic "inclusions" formed by amorphous moderately stained material. Their morphology and fine structure corresponded to that of cytoplasmic lipid droplets and they were *therefore almost certainly* the same granules which were stained by ordinary fat stains in the light microscopic specimens. In the neutrophils *practically* all these lipid droplets examined were true cytoplasmic inclusions, as no triple-layered limiting membrane could be demonstrated. They were therefore probably formed *de novo* by the cells in which they were located

Zucker-Franklin (21) and *Fegler* (11) examined the fine structure of the neutrophils from synovial and pleural effusions in patients with *rheumatoid pleuritis* or arthritis. They found the same lipid-like inclusions as described in the present material "gray globular structures" (21) or gray homogenous inclusions" (11) Some of these granules, however were limited by a distinct membrane—which *probably* indicated that they were phagocytosed material—but most granules were not. Their findings were thus consistent with the *present* findings that the lipid-like inclusions of the neutrophil granulocytes in general were not directly *formed* by *phagocytosis of lipid* droplets.

In addition to the lipid-like inclusions, the neutrophils—like macrophages—of the pleural *effusions contained* many phagocytotic vacuoles with a wide variety of phagocytosed material. Phagosomes of a similar morphology have *previously* been found in neutrophils from rheumatic effusions (11 12) and are probably the type of granules which contained *nuclear* antigen, rheumatoid factor or immunoglobulins which, on the basis of im-

mmunofluorescence microscopy have been described by several authors (1 2, 7 13 16) This gives substance to the presumption that the fat-like granules seen on both light microscopy and electron microscopy would not seem to be identical to the immune complexes. The same conclusion was also reached by *Zucker Franklin* (21) who found that the number of lipid like granules in neutrophils far exceeded the number of immuno-complex containing granules seen by immunofluorescence microscopy The aetiology of the formation of lipid like inclusions in the neutrophils of the pleural fluid is unknown. It is natural to consider their formation as a type of fatty metamorphosis, which could be due to various unphysiological conditions present in the abnormal pleural fluid. That this may be the case finds some support in the systematic alteration of the glucose concentration in the pleural fluid from these patients (10) A similar change has been observed by others in the rheumatic articular fluid (22)

The half-life of the neutrophil granulocytes with cytoplasmic lipid like granules has been found to be approximately 4 hours in rheumatic articular effusions (14) This might well explain the presence of free lipid droplets in the articular fluid. A similar mechanism could hypothetically explain—as suggested by *Bower* (4)—the opalescence of the pleural fluid in cases of rheumatoid pleuritis, and also of the pleural fluid examined here.

In our material the three types of granules normally seen in neutrophil granulocytes were not equally well preserved during preparation for electron microscopy Under different conditions some granules will often be seat of considerable extraction with a resultant electron translucent appearance (20) This may in part explain why the present neutrophils displayed no large highly electron-dense azurophilic granules, or why the large type of ellipsoid granules did not contain the crystalloids normally observed in neutrophils (20) Of the "Döhle bodies" (6)—which are accumulations of granular endoplasmic reticulum (17)—"toxic granules"—which correspond to the azurophilic granules usually identified by *Romanovsky* stains (17)—and "vacuoles frequently seen during prolonged and serious bacterial infections, only the "vacuoles" were found in the granulocytes of the patients studied here "Toxic granules" were not noted and are probably never present in the neutrophils from this type of pleural effusion. The "toxic granules" described by *Zucker-Franklin* (21) in neutrophils from joints of patients with rheumatic arthritis should rather be considered as phagosomes.

In conclusion, the fine structure of granulocytes with cytoplasmic lipid-like inclusions found in the pleural fluid was identical irrespective of the primary disease in patients with carcinoma tuberculous or rheumatoid pleuritis. The presence of the lipid like inclusions seemed to be caused by *de novo* formation inside the granulocytes as well as in macrophages, lymphocytes and mesothelial cells. The fine structure of the lipid-like inclusions gave no evidence to support the presence of immune complexes, these were probably located in the—quantitative much fewer —phagosomes of the granulocytes.

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THE EFFECT OF CYCLOPHOSPHAMIDE, METHOTREXATE AND X-IRRADIATION ON THE ULTRASTRUCTURE AND ENDOCYTIC CAPACITY OF MURINE PERITONEAL MACROPHAGES

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Rhodes, J. M., Birch-Andersen, A. & Ravn, H. The effect of cyclophosphamide, methotrexate and X-irradiation on the ultrastructure and endocytic capacity of murine peritoneal macrophages. Acta path. microbiol. scand. Sect. A, 83 445-455 1975

100 mg/kg cyclophosphamide given subcutaneously daily for 5 days and whole-body X-irradiation (500 r) had an appreciable effect on the phagocytosis of *E. coli* by mouse peritoneal macrophages. Less bacteria were ingested by cells from the treated mice as compared with control cells. In contrast, whole-body X-irradiation had a stimulatory effect on pinocytosis as compared with cells from cyclophosphamide treated mice or control mice. Morphological changes in the cells from animals treated with cyclophosphamide or X-rays were evident. The cells were larger than normal macrophages and contained large amounts of lipid and phagolysosomes and had more vacuoles as compared with control cells. Many giant cells were present in the treated mice. Treatment of mice with methotrexate for 5 days, although affecting the cells morphologically in that they were larger than normal cells and had many vacuoles did not appear to have any effect on either phagocytosis or pinocytosis. Cells from methotrexate treated animals did not contain the large quantities of lipid seen in cells from the cyclophosphamide treated and X-irradiated mice.

Key words: Cyclophosphamide; methotrexate; X-irradiation; macrophages; ultrastructure.

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Recently it has been demonstrated that cyclophosphamide treatment and whole-body X-irradiation of mice reduced the *in vitro* phagocytic capacity of their peritoneal macrophages towards *E. coli* bacteria (6). The present ultrastructural study supplements the previous investigation. Experiments have been carried out in order to investigate the effect of cyclophosphamide and X-irradiation on the morphology of macrophages from the treated mice. The capacity of the macrophages to phagocytose *E. coli* bacteria and pinocytose horse-radish peroxidase (HRP) *in vitro* was also studied.

Furthermore, treatment of mice with methotrexate was included in the study since it has been shown previously that this treatment

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stimulated cells, *i.e.* they had many vacuoles, a prominent endoplasmic reticulum and traces of endogenous peroxidase (Fig 1) A few inclusions which were characterized by a rather low density to electrons and by not being limited by a membrane were also present. These inclusions were in the same position as Sudan black stained droplets in cell smears. They are most probably lipid droplets and are referred to as such throughout this paper (Fig. 1) Some giant cells were also found. Many cells from mice treated with cyclophosphamide contained larger amounts of endogenous peroxidase in vacuoles (lysosome like structures or phagolysosomes) more lipid inclusions, and were larger in size (Fig. 2) than control cells obtained from PBS injected mice. These somewhat larger macrophages still retained many of the general characteristics of a normal stimulated macrophage, *e.g.* a prominent endoplasmic reticulum (Fig 2) It was also characteristic for the cells from treated mice that there were larger numbers of giant cells present than was normally found, but the majority of the cells were intermediate between a mature macrophage and a giant cell.

Methotrexate The cells from methotrexate treated mice were morphologically similar to control cells, although they tended to be somewhat larger There was the usual heterogeneity of cell types present in these PE cells including some giant cells. Generally cells from treated mice had many vacuoles some contained lipid inclusions and phagolysosomes (Fig. 3) but the majority did not contain as much lipid as the cells from cyclophosphamide treated mice.

X-irradiation. Cells from sham-irradiated mice showed the characteristic features of the stimulated macrophage, *i.e.* many vacuoles, a few lipid inclusions, a prominent endoplasmic reticulum and some phagocytic vacuoles containing cell debris (Fig 5) Some giant cells were also present and in general PE cells obtained from sham-irradiated mice showed considerable heterogeneity

Cells from irradiated mice contained many more phagolysosomes than the control cells and many of the treated cells were of the same size as cells from cyclophosphamide treated mice The irradiated cells contained more vacuoles but did not contain quite as many lipid inclusions as the cyclophosphamide treated cells. The irradiated cells were a more homogenous population of larger cells as compared with the sham-irradiated cells. The large number of phagolysosomes in the irradiated cells is well-illustrated in Fig 4 which shows a typical cell from this cell population.

Fig 3 A macrophage obtained from mice injected with methotrexate. This cell contains more lipid (L) and phagolysosomes (P) than the control cell in Fig 1

Fig 4 A macrophage obtained from mice which received 500 whole-body irradiation. Note the presence of many vacuoles and phagolysosomes (P)

Effect on Endocytosis

Cyclophosphamide The general impression obtained was that macrophages from cyclophosphamide treated mice did not phagocytose coli bacteria to the same extent as the control cells (cf Figs. 6 and 7) It was observed that macrophages which contained many lipid inclusions tended to show decreased phagocytosis (Fig. 6) On the other hand the engulfment process was normal corresponding to what was seen in control cells.

Cells from treated mice, although not capable of quite such extensive phagocytosis as the control cells, were perfectly capable of pinocytosing HRP concomitant with the phagocytosis of *E. coli*. Furthermore, macrophages which contained a great deal of lipid and which did not phagocytose well did, however pinocytose HRP (Fig 8) Cells which were only offered HRP also showed extensive pinocytosis despite the fact that the cells showed a typical morphological response to treatment, *e.g.* the presence of many lipid inclusions.

The control cells, on the other hand,

showed extensive phagocytosis and *pinocytosis* in the same cell In a few cases a bacterium and HRP reaction products were found in the same phagolysosome

Methotrexate The phagocytic capacity of cells from treated mice did not appear to be impaired when compared with control cells (cf Fig 9 and Fig 7) *On the* other hand treated cells which phagocytosed *E. coli* were generally those which did not contain lipid inclusions (Fig 9)

Macrophages *from methotrexate treated* mice were also capable of phagocytosing *E. coli* and pinocytosing HRP simultaneously Furthermore they ingested large amounts of HRP when offered this alone

X-irradiation Cells from sham-irradiated mice phagocytosed coli bacteria efficiently showing a normal pattern of phagocytosis (Fig 10) These cells also ingested *E coli* and HRP simultaneously

Although PE cells from X-irradiated mice were incubated with the same amount of *E. coli* they did not phagocytose *E. coli* to the *same extent as the sham irradiated cells* (cf Fig 11 and Fig 10) However some of the macrophages were still capable of ingesting many bacteria Normally cells containing large amounts of lipid did not phagocytose *E coli* extensively

Cells from irradiated mice pinocytosed HRP very efficiently and this also applied to cells which contained many lipid inclusions (Fig 12) In fact, we had the impression that these macrophages contained more *HRP reaction product than cells from any* of the other groups. Although macrophages which contained many lipid inclusions did not phagocytose *E coli* efficiently it was observed that there were cells which contained both antigens. The amount of HRP in these cells appeared to be similar to that seen in macrophages which had been offered HRP alone, indicating that impairment of phagocytosis *does not necessarily affect pinocytosis*. In order to make these results more easily accessible the most prominent features of the cells are listed in Table 1

DISCUSSION

The overall impression emerging from these studies was that mice treated with cyclophosphamide methotrexate and X-rays possessed *macrophages* which were considerably altered morphologically

One striking *feature* was *the increase in size* of the macrophages obtained from cyclophosphamide and X ray treated mice. These mice possessed a rather homogenous population of large cells which were larger than those *normally* encountered in proteose/peptone stimulated PE cells.

Methotrexate also caused an increase in the *size of some of the PE macrophages, but many were still of normal size as compared with control cells.*

PE cells from cyclophosphamide and X ray treated mice contained many more giant cells than were present in cells from mice injected with PBS. Giant cells can undoubtedly be formed by fusion of macrophages (8) *It is still* an open question as

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Fig 5 *A macrophage obtained* from mice which were sham-irradiated. This cell shows the characteristic features of a stimulated macrophage: many vacuoles, well-defined endoplasmic *reticulum* (ER) and phagocytosis of cell debris (CD)

Figs. 6–12 show macrophages which were harvested from mice treated with immunosuppressive agents *in vivo* and *E. coli* and/or HRP *in vitro*

Fig 6 A macrophage from cyclophosphamide treated mice. Note that there are only a *few* ingested bacteria as compared with the control cell (Fig. 7) Note also that this cell contains some lipid inclusions (L) and phagolysosomes (P) The endoplasmic reticulum (ER) is well-defined

Fig 7 A macrophage from a pool of cells obtained from mice injected with PBS. Note that the cytoplasm is overloaded with ingested bacteria to the extent that it is difficult to distinguish [illegible] organelles.

Fig 8 A macrophage from cyclophosphamide treated mice. The cell contains many lipid inclusions (L) and has not phagocytosed the *E. coli* which can be seen extracellularly. The cell has pinocytosed HRP as demonstrated by the reaction product (RP) in the vesicles.

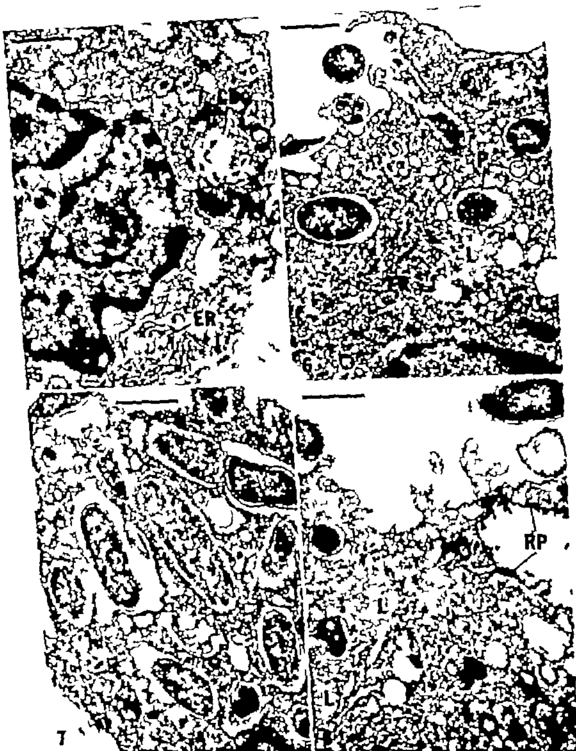

ER
P
L
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7



Fig 9 A macrophage from methotrexate treated mice This cell has phagocytosed *E. coli* efficiently note the absence of lipid inclusions.

Fig 10 A macrophage from sham-irradiated mice This cell has phagocytosed *E. coli* efficiently

to whether treatment with immunosuppressive agents can result in such a fusion and thus explain the increase in the number of giant cells. It has been suggested that alterations in the plasma membrane might facilitate fusion (8) and it is conceivable that immunosuppressive agents may have an effect on plasma membranes. Nevertheless, it is a well-known fact that alkylating agents and X rays can cause formation of giant cells (2, 9) *Castaldi et al.* (4) also observed such changes in bone mar-

TABLE 1 *The Effect of Immunosuppressive Treatment of Mice on the Morphology Phagocytic and Pinocytic Capacity of Peritoneal Macrophages*

	Non-treated controls	Cyclophosphamide	Methotrexate	Whole-body X-irradiation
Morphology	Most cells 15 20 µ	Larger cells	Cells somewhat larger	Larger cells
	Some giant cells	Many giant cells	Some giant cells	Many giant cells
	Few lipid inclusions	Many lipid inclusions	Few lipid inclusions	Many lipid inclusions
Phagocytosis of E. coli	Normal	Reduced	Normal	Reduced
Pinocytosis of HRP	Normal	Normal	Normal	Increased



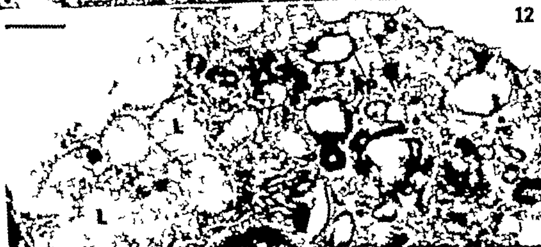


Fig. 11 A macrophage from mice which had received 500 r whole-body irradiation. This cell has not phagocytosed *E. coli* to the same extent as the control cell in Fig. 10. Note that the irradiated cell contains lipid inclusions (L) and that the endoplasmic reticulum (ER) is prominent.

Fig. 1 A macrophage from mice which had received 500 r whole-body irradiation. Large amounts of HRP reaction products (RP) indicate that the cell was capable of very active pinocytosis. Note the many lipid inclusions.

row cells after cyclophosphamide treatment.

The other striking feature of the irradiated and cyclophosphamide treated cells was the presence of many lipid inclusions and phagolysosomes in the majority of the cells. *F dork et al.* (5) induced toxic vacuoles in macrophages *in vitro* by the addition of chloroquine and found that what they called autophagic vacuoles were derived from toxic vacuoles which incorporated damaged cytoplasmic material. In our case a similar sequence of events could well apply to cells from cyclophosphamide and X-ray treated mice since many vacuoles were formed in the macrophages after treatment. Some of these might be toxic vacuoles, which later develop into what we have termed phagolysosomes.

According to *Bessis* (1) the presence of lipid is characteristic of senescent cells. The cells have a tendency to become infiltrated with various substances, particularly lipids and this is considered to be one of th

indicators of metabolic difficulty In our experiments, the presence of many phagolysosomes and lipid inclusions in PE cells from treated mice *strongly suggests that immunosuppressive agents* accelerate senescence.

Geiger & Gallily (7) have recently observed, by scanning electron microscopy that macrophages from mice irradiated with 500 r contained many more "hole-like" features on their surfaces than normal cells. They suggest that these holes may represent defects in or openings of phagocytic and pinocytic vesicles. *It is also a possibility that the holes* might represent vacuoles of the type present in abundance in our sectioned cells.

Geiger & Gallily (7) have also demonstrated that an activated state exists in irradiated macrophages, *i.e.* as regards pinocytosis, DNA synthesis and enzyme synthesis. We could confirm these findings as we had the impression from the sections that such cells *had taken up more HRP and in addition* we found that irradiated macrophages ingested more [125]I HRP than control cells. Furthermore, the level of acid phosphatase was higher in irradiated macrophages (unpublished observations)

It was recently shown (6) that the phagocytic ability of macrophages from mice treated with cyclophosphamide and X rays *was impaired. Our experiments confirm* the previous results, although one should bear in mind that normally fewer cells are examined in ultrastructural studies.

In order to explain the observed decrease in phagocytic activity it was suggested (6) *that cyclophosphamide treatment and irradiation could prevent the normal influx of* cells from the bone marrow because they are anti-proliferative agents (3) However these agents might also kill a proportion of the cells already present in the peritoneum or in other words select out a subpopulation of peritoneal cells corresponding to one of the populations *of macrophages which can be* obtained by fractionation on a density gradient (11 12) The cells which survive may still retain their capacity to phagocytose, but be so active in ingesting cell debris that they are unable to engulf *E. coli* efficiently It was also observed in the present experiments that more HRP was pinocytosed by *cells from* X-irradiated mice than by cells from control mice. Therefore one could ask whether the uptake of large amounts of cell debris or other material released following irradiation stimulates macrophages to become more actively pinocytic despite the fact that phagocytosis is impaired.

We would like to acknowledge the excellent technical assistance of *A. G. Overgaard* for the photographic work and *F. Larsen* for mounting the illustrations.

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Acta path. microbiol. scand. Sect. A, 83: 454–466, 1975

TIME COURSE OF CHANGES IN PLASMA RENIN AFTER BLOCKADE OF THE RENIN SYSTEM

Studies of Conscious and Anaesthetized Normal, Adrenalectomized and Spontaneously Hypertensive Rats

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Inhibition of the angiotensin I converting enzyme with SQ 20.881 results in a 2 to 35 fold increase in plasma renin concentration in normal rats and in spontaneously hypertensive rats. The effect is transient, lasting for 1 to 3 hours even in the presence of prolonged blockade. The relative increase is independent of the pretreatment plasma renin concentration. The blood pressure is unchanged in conscious rats in which the effect of SQ 20.881 on plasma renin is believed to be due to a blockade of the negative feedback of angiotensin II on renin release. In anaesthetized rats, SQ 20.881 has an additional hypotensive effect which augments the increase in plasma renin. Saralasin is without effect on blood pressure and plasma renin in conscious normal rats and in spontaneously hypertensive rats, while it causes a transient 3 to 27 fold increase in plasma renin concentration in anaesthetized rats. It is suggested that this increase is hardly due to an interception of the feedback, but to the concomitant fall in blood pressure as a similar hypotension and increase in plasma renin is produced by dihydralazine. It is furthermore found that Saralasin blocks renin release induced by SQ 20.881. This demonstrates that Saralasin is bound to the receptors in the juxtaglomerular cells and has slight agonistic properties there. Both in conscious rats and in anaesthetized adrenalectomized rats substituted with DOCA and salt, SQ 20.881 as well as Saralasin causes transient increases in plasma renin concentration. If such rats are only substituted with salt and not with DOCA, the effects of both blockers are in the form of severe hypotension and a permanent elevation of plasma renin.

Key words: Renin, plasma; time course of changes; renin-system blockade.

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Continuous intravenous infusion of angiotensin II is known to reduce renin release in dogs, man, sheep and rats (for literature see *Page & McCubbin* 1968) probably by a negative feedback mechanism. In accordance with this, it was found that blockade of the renin system, either with a converting enzyme inhibitor or with an angiotensin II blocker results in an increase in plasma renin in dogs

with acute renal one-kidney hypertension or with thoracic inferior vena cava constriction, as well as in dogs on low salt diet and in adrenalectomized dogs, but not, or only slightly in normal dogs (*Miller et al* (1972) *Johnson & Davis* (1973) *Samuels et al.* (1973) and *Ayers et al* (1974)) In conscious *rabbits* treatment with an angiotensin II blocker resulted in an increase in plasma renin whether the animals were on a normal or a sodium-deficient diet (*Steele & Lowenstein* 1974) In conscious rats injection of a converting enzyme inhibitor (the synthetic nonapeptide >Glu Trp-Pro-Arg Pro-Gln-Ile Pro-Pro, designated *SQ 20.881*) is followed by a rapid, marked increase in plasma renin both in normal and adrenalectomized animals substituted with DOCA and salt. Contrary to this, infusion of the competitive angiotensin II inhibitor 1-Sar-8-Ala Ang II designated *Saralasin* or P 113 only resulted in a marked increase in plasma renin in adrenalectomized rats, but not in conscious normal rats (*Bing* (1973) and *Oates et al.* (1974)) Increase in plasma renin after infusion of Saralasin has also been observed in *patients with hypertension* in whom it was found that the degree of renin stimulation did not seem to correlate with the duration of the infusion, nor did it seem to depend on blood pressure reduction (*Brunner et al.* 1973 *Gavras et al.* 1974)

The aim of the present study was to obtain further information of the effect of these two differently acting blockers of the renin system, using conscious and anaesthetized normal adrenalectomized and spontaneously hypertensive rats. The time course of the effect of the blockers on plasma renin was compared with that of angiotensin II. For further comparison with the effect of the blockers which decrease the blood pressure of anaesthetized rats, studies were performed with a view to averaging the effect on blood pressure and renin of treatment with dihydralazine, a hypotensive drug which appears to act by reducing the tone in arterioles, with some depression of vasomotor centre activity and which increases renal blood-flow

MATERIAL AND METHODS

Animals Female Wistar S.P.F rats and rats with spontaneous hypertension (SHR/N) obtained from the Nat. Inst. Health, Maryland, U.S.A., who had received the strain from Professor Okamoto Kyoto, Japan. Their weight was about 200 g. *Anaesthesia* either 100 mg/kg b.w of amobarbital (amytal) or in most rats, 150 mg/kg b.w of the thiobarbiturate Inactin (Promonta, Hamburg) i.p., the type of anaesthesia being without influence on the results. Some rats were *conscious* catheters had been inserted in the carotic artery and jugular veins during a short-term ether anaesthesia at least 1½ hours before the start of the experiment. Some rats were *ad renalectomized* and substituted with 1 per cent sodium chloride solution as drinking fluid and 0.5 mg desoxicortone acetate (DOCA) given s.c. in all on the first 4 days of the week the dose being trebled on the 5th day while no injections were given during the following 2 days. In some of these rats, the treatment with DOCA was stopped after 2 weeks, the treatment with sodium chloride being continued for a further 3 weeks or more.

Blood pressure was recorded using Tybjærg Hansen transducer and a Servogor 511 recorder

The effect as well as the *degree of the blockade of the renin system* were measured by determination of the ratio between the pressor responses of angiotensin I and of angiotensin II before and after treatment with SQ 20.881 (Squibb Corp Princeton) The ratio between angiotensin II and noradrenalin was used when Saralasin, (Norwich Pharm. Comp., New York) was infused. These ratios were constant or only slightly changed when no blocker was given. *SQ 20.881* was given by a single injection of 2 mg/kg b.w., which in specified experiments was followed by infusion of 1.25 mg/kg/hour *Saralasin* was given by infusion of 0.6 mg/kg/hour *Angiotensin II* was infused in a dose of 14 µg/kg/hour The volume infused was from 0.23 to 1.2 ml per hour The blockers and the angiotensin were dissolved in 0.9 per cent saline or 5 per cent glucose, the result being independent of the solvent used. In most cases, *Dihydralazin* (Nepresol® Ciba) was given by a single injection of 100 to 400 µg/kg, which in some rats was followed by continuous infusion of 100 to 600 µg/kg/hour

Plasma renin concentration was determined in plasma from 75 µl samples of blood drawn from the carotic or femoral artery using the capture radioimmunoassay for angiotensin I as described by *Poulsen & Jørgensen* (1974) The concentration was expressed in Goldblatt units (GU) × 10⁻⁴ × ml by comparison with the *standard human renin* preparation obtained from Division for Biol. Standards, Holly Hill, London, donated by Dr *Haas*, Cleveland, U.S.A. The *angiotensin II* was the

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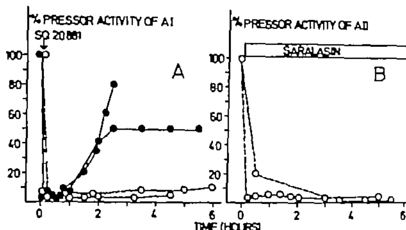


Fig 1 Pressor sensitivity to angiotensin after blockade A Effect with time on the sensitivity to angiotensin I of a single i.v injection (arrow) of 2 mg/kg b.w SQ 20.881 (●——●) and of the same dose followed by continuous infusion of 1.2 mg/kg/h (○- - -○) *B.* Effect with time on the sensitivity to angiotensin II of continuous i.v infusion of 0.6 mg/kg/h Saralasin.

Holly Hill Standard 65A, and the *angiotensin I* was a preparation purchased from Beckman, and standardized against research Standard angiotensin I from Holly Hill

RESULTS

I Changes with Time of the Pressor Effect of Angiotensin after Blockade of the Renin System

A. *Inhibition of the converting enzyme* was obtained by a single injection of *SQ 20.881* which in some experiments was followed by continuous infusion of this nonapeptide. After a single i.v injection of 2 mg/kg b.w (N = 7) the sensitivity to angiotensin I rapidly fell to less than 10 per cent of the original and stayed at this low level for about an hour thereafter rising to the pretreatment level which was reached in about 1½ to 2 hours (representative experiments in Fig 1A) When a single injection of 2 mg/kg was followed by continuous infusion of 1.25 mg/kg/h (N = 4) there was a rapid decline to values below 5 to 10 per cent and the effect lasted for at least 6½ hours (Fig 1A)

B. *Inhibition of the pressor effect of angiotensin II* with the specific competitive antagonist *Saralasin* was obtained by continuous infusion of 0.6 mg/kg/h (N = 4) which resulted in a rapid fall in sensitivity to values below 5 to 10 per cent, the sensitivity thereafter being less than 5 per cent for at least 5¼ hours (Fig 1B)

II Plasma Renin Concentration with Time in Normal Rats (Controls without Blockade of the Renin System)

The plasma renin concentration according to time was studied in 4 conscious and 6 anaesthetized Wistar rats, four of which were continuously infused with a saline solution or a 5 per cent glucose solution in the same volume per hour as that used in the following experiments with infusion of blockers. The start values were between 0.1×10^{-3} and 1×10^{-3} G.U. $\times$ ml^{-1} and the values fell to below 0.5 $\times 10^{-3}$ G.U. $\times$ ml^{-1} during the following 6 hours, this being the case in all rats whether infused or not (Fig 2A)

III Changes in Plasma Renin Concentration after Inhibition of the Converting Enzyme with the Nonapeptide SQ 20 881

A. *Normal Wistar rats.* In 14 conscious rats, the injection of *a single dose of 2 mg/kg b.w of SQ 20.881* was followed by a rapid, marked increase in renin concentration. In about 30 minutes it rose, on an average by 5 fold (range 2–16) independent of the pre-

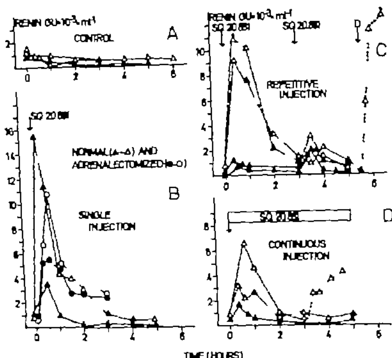


Fig 2 Effect of SQ 20 881 on plasma renin concentration with time A. Controls. Plasma renin concentration, which in this and the following figures is given in Goldblatt units (G.U.) $\times 10^{-4} \times ml^{-1}$ with time in 1 conscious (▲) and 2 anaesthetized (△) normal rats, one of which had continuous infusion of 1.5 ml/h of a 0.9 per cent NaCl solution. This is the maximum volume of fluid used for infusion of the blockers *B* Effect on plasma renin with time of *single injection* of mg/kg SQ 20.881 in representative conscious (▲) and anaesthetized (△) normal rats, and in conscious (●) and anaesthetized (○) adrenalectomized rats substituted with DOCA and salt. *C* Effect on plasma renin of *repetitive injections* of 2 mg/kg SQ 20.881 on conscious (▲) and anaesthetized (△) normal rats. One rat received 15 mg/kg dihydralazine at the end of the experiment (△ △) *D* Effect on plasma renin of injection of 2 mg/kg, followed by *continuous infusion* of 1.25 mg/kg/h of SQ 20.881 in conscious (▲) and anaesthetized (△) normal rats. One rat received 4 mg/kg dihydralazine (△ △) 3 hours after the start of the infusion.

treatment level. The concentration returned close to the starting value about one hour after the injection and stayed there, or was still lower for the following hours (▲ Fig. 2B) In 9 of the 14 conscious rats, a *second injection* of the same dose of the inhibitor was given 3 hours after the first and caused a new increase in plasma renin which increase was about the same or somewhat higher and more prolonged than the first (Fig. 2C) The renin concentration rose in about 30 minutes to about 7 fold (range 3 16) the value found immediately before the second injection. Most values were still elevated 1 to 2 hours after the injection. In a further 8 conscious rats, the single injection of 2 mg/kg was *followed by continuous infusion* of 1.25 mg/kg/hour which, however did not prolong the transient increase in plasma renin (▲ Fig 2D)

The effect of SQ 20.881 was also studied in *amytal or inactin anaesthetized rats.* In 21 normal rats which received a *single injection* of 2 mg/kg of the inhibitor there was a rapid, marked increase in plasma renin concentration, reaching its maximum after about 15 to 30 min. The average increase was higher and more prolonged in these anaesthetized (△ Fig 2B) *rats than in* the conscious animals, the mean maximal renin concentration being 20 fold (range 6–35) the pretreatment value. The concentration was

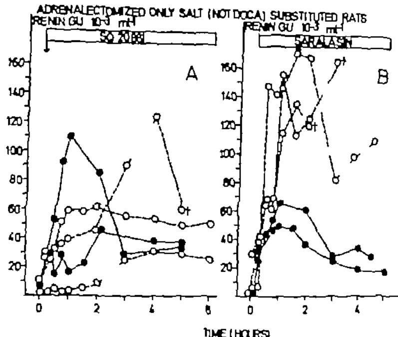


Fig 3 Effect of SQ 20.881 (A) and of Saralasin (B) on plasma renin concentration with time in salt substituted adrenalectomized rats. In both groups (A and B) the adrenalectomized rats were substituted first with DOCA and salt for 2 weeks and thereafter for at least 3 weeks *only with salt*. The conscious (●) or anaesthetized (○) rats were treated either (A) with injection of 2 mg/kg followed by continuous infusion of 1.2 mg/kg/h of SQ 20.881 or (B) with 0.6 mg/kg/h Saralasin. † marks that the rat died spontaneously. Note the high renin values on the ordinates.

still markedly elevated one and two hours after the injection, but after three hours nearly *all* values had returned to the level found at the start of the experiment. Five of these unanaesthetized rats received a *second injection* of the same dose of the inhibitor 3 hours after the first. This caused a new rise in plasma renin, which, however only rose to about twice (range 1–3 fold) the value found immediately before the second injection (Fig 2C). Five hours and a half after the start of the experiment, 3 rats which had previously been injected twice with SQ received an injection of dihydralazine which resulted in a rapid and pronounced increase in plasma renin concentration (△ △ Fig 2C). A decreased effect with time of the inhibitor on plasma renin was also found in 8 out of 9 *rats which had been anaesthetized with inactin for 4 to 5 hours prior to the injection* of the inhibitor. In these rats the mean rise was 5 fold (range 2–10). A further 4 anaesthetized rats received the *single injection* of 2 mg/kg *followed by continuous infusion* of 1.25 mg/kg/h or in one rat, 7.5 mg/kg/h. In these rats there was the *same* transient effect on the renin concentrations with a 5 to 11 fold increase peaking after 20 to 40 minutes. The concentration returned close to the starting values after 2 hours and stayed at the level in spite of the continued infusion (△ Fig 2D). Two of the rats which had injections of dihydralazine at a time when the renin concentration had returned to normal values responded with a 6-9 *fold-increase* in renin concentration (△ △ Fig 2D).

B *Adrenalectomized rats substituted with DOCA and salt.* Five conscious and 3 anaesthetized *adrenalectomized* rats substituted with DOCA and salt reacted to a single dose of 2 mg/kg SQ 20.881 in a similar way as normal rats (● and ○ Fig 2B). There was, however *a tendency to a more prolonged* elevation of plasma renin in the adrenalectomized animals.

Contrasting with the transient increase in

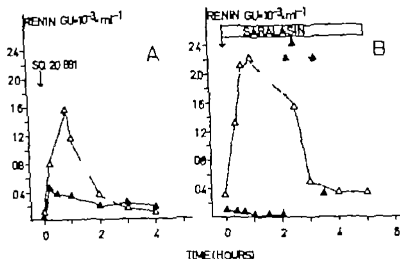


Fig. 4. Effect of SQ 20.881 (A) and of Saralasin (B) on plasma renin concentration with time in spontaneously hypertensive rats. The conscious (▲) or anaesthetized (△) rats were treated either (A) with injection of 2 mg/kg SQ 20.881 or (B) with 0.6 mg/kg/h Saralasin. △ △ marks the result of treatment with dihydralazine. Note the low renin values on the ordinate.

plasma renin to occur after blockade in all other groups of rats studied, the increase was persistent in two conscious rats and in four anaesthetized adrenalectomized rats which after 2 weeks of substitution therapy with DOCA and salt for the next 3 weeks were ly substituted with 1 per cent sodium chloride as drinking fluid (Fig. 3A). All but one these rats started with an elevated plasma renin concentration of about 5×10^{-4} G U per ml plasma. After infusion of SQ 20.881 the *relative* increase in renin was about the same as that in normal rats and thus, values as high as 125×10^{-4} G U × ml^{-1} were measured.

C. *Spontaneously hypertensive rats.* Four *conscious* spontaneously hypertensive rats of the Okamoto strain which had significantly lower pre-injection plasma renin concentration (about 0.05 to 0.2 G U × 10^{-4}) than normal Wistar rats reacted to a single dose of 2 mg/kg *SQ 20.881* with about the same *relative* increase in plasma renin as that found in normotensive rats, the values rising by about 7 fold the start value in three animals and by 30 fold in the fourth. The maximal value was reached after 15 to 20 minutes after which there was a decrease the renin concentrations, however still being somewhat elevated 2 to 4 hours after the injection (▲ Fig. 5A).

The reaction was rather similar but more pronounced in 5 similarly treated inactin *anaesthetized* spontaneously hypertensive rats in which the renin concentration reached a value of about 10 fold (range 6–16) the start value about 40 minutes after the injection. As in the conscious rats, the response was transient, but the start values were not reached until after 3–4 hours (△ Fig. 5A).

IV. Changes in Plasma Renin Concentration with Time after Blockade of Angiotensin II with Saralasin

A. *Normal Wistar rats.* In 7 conscious rats which received *continuous infusion of 0.6 mg Saralasin/kg/h* for from 1½ to 3 hours, there was no effect of the blockade on plasma renin which stayed at normal concentrations between 0.1 and 0.5 × 10^{-4} G U (▲ Fig. 5). In four conscious, *normal* rats which were treated with the same doses of DOCA and salt as those used for substitution of the adre

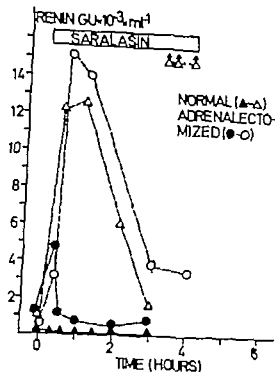


Fig 5 Effect of Saralasin on plasma renin concentration with time Effect of continuous infusion of 0 6 mg/kg/h Saralasin in conscious (▲) and anaesthetized (△) normal rats and in conscious (●) and anaesthetized (○) adrenalectomized rats substituted with DOCA and salt. One of the rats received an injection of dihydralazine (△ △) at the end of the experiment.

nalectomized rats (described in section IV B) the start values of plasma renin were below the detection limit of 0 03 × 10⁻³ G U × ml Treatment with Saralasin did not bring the concentration above this limit.

When the Saralasin infusion was given to 20 *anaesthetized* rats there was a rapid, transient 13 (range 3–27) fold increase in plasma renin. The rise was significant already 10 min. after the start of the infusion and reached its maximum value after 30 to 60 minutes. The values reached starting levels after 3–4 hours and stayed there for at least 6 hours in spite of the continued infusion (△ Fig 5) Three rats which received an i.p injection of 12 mg/kg *dihydralazine* (Nepresol®) at a time when they no longer responded to the continuous infusion of Saralasin, reacted with rapid, enormous increases in plasma renin which reached concentrations of 27 to well over 50 × 10⁻³ G U /ml (△ △)

B. *Adrenalectomized Wistar rats substituted with DOCA and salt.* Continuous infusion of *Saralasin* to 5 *conscious adrenalectomized* rats was followed by a 2 to 20-fold transient increase in plasma renin concentration, the maximum value being reached after 15 to 30 minutes upon which the values fell, reaching the start value and staying there for the following period although the infusion was continued (● Fig. 5) Similar but more pronounced increases were found in 7 *anaesthetized* adrenalectomized rats in which the maximum value of about 20 fold (range 4 to 36) the start concentration was reached later than that found about an hour after the start in the conscious animals. Although there was a marked fall in the values during the following hours of continued infusion, the period of time in which increased values were observed was more prolonged (○ Fig. 5) than in the conscious rats.

In 2 conscious and 3 anaesthetized adrenalectomized rats which first were substituted with DOCA and salt for 2 weeks and thereafter only received salt substitution during the following 3 to 5 weeks, the Saralasin infusion—exactly as SQ 20.881—produced changes in plasma renin concentration which markedly differed from those found in all the other groups of rats (Fig. 3B) These rats 1) had an abnormally high pretreatment renin concentration, 2) reached extremely high concentrations (maximum about 190 G U × 10⁻³ × ml) during the treatment, 3) had increased values for a long period of time values which were still markedly increased up to 5 hours after the start of the Saralasin infusion. 4) Several animals died spontaneously probably due to the pronounced fall in blood pressure, as mentioned in the following section VI.

C. *Spontaneously hypertensive rats.* Continuous infusion of Saralasin in 7 *conscious*, spontaneously hypertensive rats showed that the plasma renin concentration was as uninfluenced in these (▲ Fig. 4B) as in conscious normal Wistar rats. Two of these rats, which

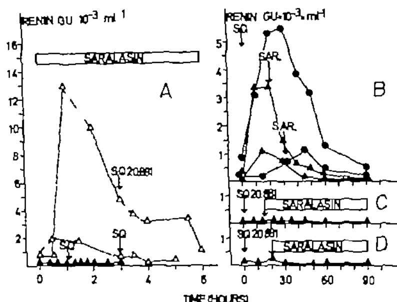


Fig 6 Changes in plasma renin concentration with time after combined treatment with SQ 20.881 and Saralasin. A During an infusion of 0.6 mg/kg/h Saralasin, a single injection of 2 mg/kg SQ 20.881 was given (arrows) to conscious (▲) and anaesthetized (△) normal rats. *B.* Subsequent to an injection of 2 mg/kg SQ 20.881 a continuous infusion of 0.6 mg/kg/h Saralasin was started after the peak values were achieved (arrows) in two conscious normal rats (▲) which responded to the infusion of Saralasin with a decrease in the elevated renin values, more rapid than that found in the two control rats (●) *C and D* Subsequent to an injection of 2 mg/kg of SQ 20.881 and before it had provoked a rise in plasma renin, a continuous infusion of 0.6 mg/kg/h Saralasin was started (arrows) in two conscious normal rats. This resulted in a total inhibition of the increase in plasma renin otherwise to be found after injection of SQ 20.881

received an injection of 12 mg/kg dihydralazine two hours after the start of the infusion of Saralasin, reacted with marked increases (30 and 22 fold, respectively) in plasma renin (▲ ▲ Fig 4B) After the infusion of Saralasin to 5 *anaesthetized* spontaneously hypertensive rats, the relative increase in plasma renin was almost the same as that found in normal rats, the maximum value being about 5 (range 3–7) fold higher than the start value (△ Fig. 4B) As in normal rats there was already after 10 minutes a steep rise in plasma renin which after about 1 to 1½ hours reached the maximum value The response was transient in spite of the continued infusion. The subsequent fall in the renin values was somewhat more prolonged in these than in normal rats.

V Changes in Plasma Renin Concentration after Combined Treatment with SQ 20.881 and Saralasin

A. *The effect of injection of SQ on plasma renin in rats 10 minutes to 3 hours after the start of continuous infusion of Saralasin* was studied in 4 conscious rats and in 3 Inactin anaesthetized rats. In the conscious rats there was as usual no effect of the infusion of Saralasin while the plasma renin in anaesthetized rats as usual was markedly elevated (Fig. 6A) In all 7 Saralasin treated rats, the injection of SQ 20.881 *failed* to give the marked increase in plasma renin otherwise seen in non-Saralasin-pretreated rats (Fig. 2B)

B. *The effect of infusion of Saralasin starting from 14 to 30 minutes after a single injection of SQ 20.881* was studied in 6 con-

scious rats. Six other conscious rats which served as controls only received the primary dose of SQ 20.881 The blood samples were taken at short intervals during the short term effect of SQ 20.881 In 3 rats, the increase in plasma renin caused by injection of SQ 20.881 had peaked before the start of the infusion of Saralasin which markedly shortened the response to SQ 20.881 as compared with the duration in the controls (Fig. 6B)

In the other 3 rats, the infusion of Saralasin was started early in the SQ 20.881 response before the plasma renin had started to rise. The usual increase in renin was here

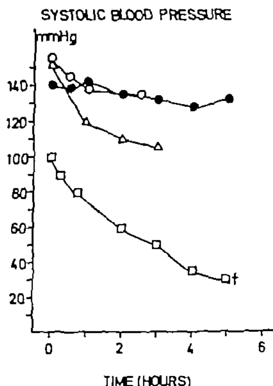


Fig 7 Effect of the blood pressure with time of the standard doses used for infusion of SQ 20.881 or Saralasin Curves representing conscious normal rats or adrenalectomized rats substituted with DOCA and salt given either of the blockers (●) curves representing anaesthetized normal rats receiving SQ 20.881 (○) or Saralasin (△) and curves representing conscious or anaesthetized adrenalectomized rats substituted only with salt and treated with either SQ or Saralasin (□) † indicates spontaneous death.

totally inhibited by the Saralasin infusion (Fig 6C and D)

VI Influence of Blockade of the Renin-System on the Blood Pressure

The influence of blockade of the renin system on the blood pressure was studied in 47 rats. In 11 out of 16 *conscious*, normal or adrenalectomized rats substituted with DOCA and salt there was no significant change in the systolic blood pressure whether they were treated with SQ 20.881 or Saralasin. Two normal rats treated with SQ reacted with a pressor increase of 40 and 50 mm, respectively while 2 adrenalectomized rats and 1 normal treated with Saralasin, reacted with a pressor increase between 3 and 22 mm. This agonistic response was in all cases of only 2 to 3 minutes duration and none of the 16 conscious rats reacted with a depressor response (Fig 7)

The influence on the blood pressure in *anaesthetized* normal rats differed in two ways from that in the conscious rats 1) all anaesthetized rats reacted with a pressor response which in most cases was about + 15 mm, lasting for from 1 to 3 minutes 2) all of the anaesthetized rats reacted with a depressor effect, immediately following the short pressor effect. In 4 normal rats treated with SQ, the blood pressure decreased 10 to 15 mm in 2 rats which had a pretreatment blood pressure of 120 and 175 mm, respectively while it fell gradually by 20 and 40 mm in the 2 other rats which had pretreatment pressures of 155 and 170 mm, respectively In 4 Saralasin treated rats the blood pressure decreased from 26 to 45 mm, reaching these low values after about 1½ hour thereafter slowly increasing in one case it reached the start level 4¼ hours after the start of the infusion. In 2 anaesthetized, spontaneously hypertensive rats with blood pressures of 155 and 175 mm, the fall was still more pronounced reaching values of 110 to 120 mm and staying at this level throughout the 3 hours during which Saralasin was infused. The curves representing the blood

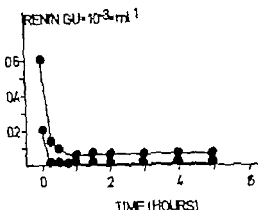


Fig 8 Effect on plasma renin concentration with time of continuous infusion of 14 μg/kg/h angiotensin II in two normal rats.

pressure in the adrenalectomized rats (Fig 7) which were substituted only with salt for at least 3 weeks after a previous substitution with DOCA and salt, differed from all curves previously mentioned. The pretreatment level was about 110 Hg Injection of SQ in a dose of 2 mg/kg, followed by infusion of 1.25 mg/kg/h, resulted in a short pressor effect of about 55 mm in 2 conscious rats and in 3 anaesthetized rats, while such response lacked in the 3 (one conscious, two anaesthetized) Saralasin treated rats in which the blood pressure was followed. Still, during infusion of SQ as well as Saralasin, all but one of these rats reacted with a pronounced decrease in blood pressure to values which finally were as low as from 20 to 45 mm Hg in most cases. This severe hypotension was probably responsible for the spontaneous death of several of these rats (Fig. 7)

VII Changes in Plasma Renin and in Blood Pressure after Continuous Infusion of Angiotensin II

The effect of continuous infusion of angiotensin II in a dose of 14 μg/min/kg body weight was the same in 3 conscious and 4 anaesthetized normal rats. In all cases, the plasma renin fell rapidly from a pretreatment value which varied from 0.1 to 1.3 G U × 10 × ml to a value below 0.03 to 0.1 G U $\times 10^{-3} \times ml^{-1}$ which was reached after 30 to 60 minutes and stayed at this low level for at least 6 hours (Fig 8) This constant pattern of the changes with time in plasma renin was independent of the effect of the angiotensin II infusion on the blood pressure which differed in the 4 rats in which it was measured. There was in all cases a rapid rise in blood pressure which rose by about 30 to 50 mm. Thereafter it fell slowly to about 5 mm above the pretreatment value in 2 cases, while the fall was only from +48 mm to +30 mm in the third case in the fourth rat, the blood pressure fell from +40 to +15 after 1½ hours infusion and, after 4 hours, it rose again to a maximum of +30 mm above the pretreatment level.

VIII Changes in Plasma Renin and in the Blood Pressure after Treatment with Dihydralazine

The effect of dihydralazine was studied in 7 rats, 3 of which received a single injection of 100–190 μg/kg, while 4 received a primary injection of 200–450 μg/kg followed by infusion of 300–600 μg/kg/h. In all cases there was a dose dependent decrease in blood pressure and increase in plasma renin concentration, but although these changes ran parallel in the beginning, the time course of the changes in blood pressure and renin differed from case to case. These differences were found to be independent of the mode of administration of dihydralazine, i.e. whether it was given as a single injection or by continuous infusion. In some rats, the changes continued to run parallel either both returned to the pretreatment values (Fig. 9A) or both stayed at the changed level (low blood pressure and high plasma renin) as seen in Fig. 9B. In most rats, however the plasma renin decreased from its elevated level while the blood pressure was still low (Fig 9C) In one such experiment (Fig 9C) a doubling of the dose of dihydralazine resulted in a further decrease in blood pressure to values below 70 mm Hg as well as in a secondary marked rise in plasma renin.

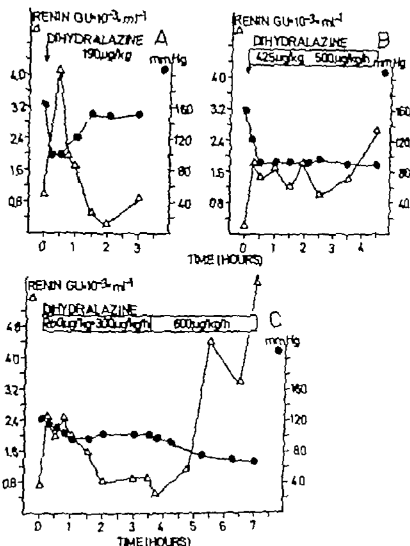


Fig 9 Effect of dihydralazine on blood pressure and plasma renin concentration with time. A Effect of a single injection of 190 µg/kg on systolic blood pressure in mm Hg (●) and plasma renin (Δ). B. Effect of injection of 425 µg/kg followed by 500 µg/kg/h. C Effect of 260 µg/kg followed by infusion of 300 µg/kg/h, the dose being increased up to 600 µg/kg/h after 3½ hours' infusion.

DISCUSSION

The decrease in plasma renin found after intravenous infusion of angiotensin II has also been found in the non-filtering kidney model (*Shade et al.* 1973) and is therefore believed to be mediated by a vascular receptor in the renal afferent arteriole or by a direct influence on the juxtaglomerular cells. In the present experiments on normal Wistar rats, injection or infusion of the *converting enzyme inhibitor SQ 20.881* resulted in a marked decrease in angiotensin II formation (Fig 1A) and therefore also in an increase in plasma renin (for literature see the introduction). When only a single injection was given, the decreased angiotensin formation was of limited duration (Fig 1A) and it is therefore understandable that also the increase in plasma renin was of almost equally short duration (Fig 2B). The increase in plasma renin was much more pronounced in anaesthetized rats than in conscious rats (Fig 2B) probably due to the difference in

the effect of the injection of SQ on the blood pressure which remained unaltered in the conscious animals, but was lowered in the anaesthetized rats (Fig. 7). When a second injection of SQ was given about 3 hours after the first, the conscious rats reacted with a new increase in plasma renin which was more pronounced than the first (Fig. 2C) which may be due to a summation of the effect of a small, in itself inactive remainder of the previously injected dose and of that of the new injection of the blocker. The fact that quite the opposite was the case in anaesthetized rats in which the second injection gave a markedly smaller increase in renin than the first (Fig. 2C) is explained by the decreased response to the blocker after prolonged anaesthesia. While the limited duration of the response of plasma renin corresponded to the limited duration of the inhibition of conversion of angiotensin I to angiotensin II it was surprising that a similar short term increase in plasma renin was found after continuous infusion of the blocker (Fig. 2D)—this being the case even if a 6-fold higher dose had been used—although the infusion results in a lasting blockade of the conversion of angiotensin I (Fig. 1A). This was not due to a total lack of ability to release renin, as shown by the effect of injection of dihydralazine (Fig. 2D). The transient effect on renin release of continuous infusion of the converting enzyme inhibitor contrasts with the effect of continuous infusion of angiotensin II throughout at least 6 hours (Fig. 8). A transient increase in plasma renin similar to that seen after continuous infusion of the converting enzyme blocker was found in some (Fig. 9C) but not all rats (Fig. 9B) continuously infused with the hypotensive drug hydralazine.

Treatment with *the angiotensin II analogue Saralasin* is not, as the blockade with inhibition of the converting enzyme, followed by a decrease in plasma angiotensin II but works by binding to the angiotensin II receptors and thereby competing with the effect of AII. This well-known binding of Saralasin to the angiotensin II receptors in arterioles and adrenals was also found to take place in the renin releasing juxtaglomerular cells in the kidney. This could be shown by combined treatment with the two differently acting blockers, both when Saralasin was infused before the SQ 20.881 was injected (Fig. 6A) and in experiments in which the injection of SQ was given before Saralasin had been infused (Fig. 6B, C, D). In both cases, Saralasin was found to prevent the increase in plasma renin otherwise to take place when plasma angiotensin II is lowered after injection of SQ 20.881. This means that Saralasin not only binds to the AII receptors but also exhibits slight agonistic properties on the juxtaglomerular cells.

Injection of Saralasin in conscious rats (Fig. 5) does not result in any increase in plasma renin or change in blood pressure. This is in agreement with the abovementioned interpretation, since slight agonistic properties will mimic the effect of angiotensin II on the juxtaglomerular cells.

Besides using normal Wistar rats, studies were also performed with rats of the spontaneously hypertensive strains Okamoto strain and with adrenalectomized Wistar rats.

Rats with *spontaneous hypertension* in which plasma renin was significantly lower than in the normal Wistar rats reacted in the same way as the normal rats, the *relative* increase and the time course of changes in plasma renin being identical in the two groups. This was the case when SQ 20.881 was given to conscious or anaesthetized rats and when Saralasin was given to anaesthetized rats. Exactly as in normal rats, Saralasin was without effect on plasma renin in conscious, spontaneously hypertensive rats.

In adrenalectomized Wistar rats substituted with DOCA and salt the effect on plasma renin of blockade of the converting enzyme was the same as in normal rats, apart from a tendency to a somewhat prolonged time course of the effect (Fig. 2B). This similarity in response was also found when the blockade of the renin system was brought about by infusion of Saralasin into *anaesthetized* normal and adrenalectomized rats. But, contrary

to conscious normal rats which do not react to treatment with Saralasin *conscious* adrenalectomized rats respond with about the same increase in plasma renin (Fig 5) otherwise found after treatment with SQ 20881 This difference between normal and adrenalectomized rats cannot be explained by any difference in the effect of Saralasin on the blood pressure which was unaltered in both groups of conscious rats (Fig 7)

Still another pattern of the time course of the changes in plasma renin after blockade of the renin system was found in *adrenalectomized Wistar rats substituted only with salt* These rats which all but one had markedly increased plasma renin levels reacted in about the same way whether they were treated with SQ 20.881 (Fig 3A) or with Saralasin (Fig 3B) and whether they were conscious or anaesthetized. The primary increase in plasma renin was *relatively* the same as that seen in normal and in spontaneously hypertensive rats and thus, very high renin concentrations were reached. Contrary to all the other rats studied, however response in these rats was markedly prolonged and thus, increased plasma renin values could be found even 5 hours after the start of the infusion of SQ or Saralasin This prolonged effect is probably caused by the prolonged, very marked fall in blood pressure, a fall that even increased with time resulting in degrees of hypotension of such magnitude that several of these rats died (Fig 7)

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A MORPHOMETRIC STUDY OF NORMAL HUMAN LIVER CELL NUCLEI

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Ranek, L., Keiding, N. & Jensen, S. T. A morphometric study of normal human liver cell nuclei. Acta path. microbiol. scand. Sect. A, 83: 467–476, 1975.

Using the projecting method, measurements were made of the size of nuclei of liver cells in liver biopsies taken from ten patients with normal liver histology and normal liver function tests and from two patients with acromegaly. For the statistical analysis a parametric model of the distributions of the nuclear radii, the χ distributions, was used, making an estimation of the frequencies of di-, tetra- and octaploid nuclei possible. The distribution of the lengths of the nuclear radii was consistent with normal distributions corresponding to the different ploidy classes. Diploid nuclei constituted 84 to 99 per cent of the liver cell nuclei, and the frequency of polyploid nuclei increased with increasing age. Samples of nuclei within the same biopsy revealed significant differences in the mean radius of diploid nuclei and in the frequency of polyploid nuclei. This biological variation must be taken into account in the interpretation of karyometric data. One of the patients with acromegaly had a higher frequency of polyploid nuclei than seen in normal patients, and in both patients with acromegaly the size of diploid nuclei was large compared with normals. The frequency of binuclear nuclei was unrelated to age and frequency of polyploid nuclei, but males were found to have more binuclear liver cells than females.

Key words: Liver cell nuclei; morphometric study.

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Classification of nuclei according to their volume was suggested by *Jacobj* (8) on the basis of his measurements of nuclear size in histological specimens from various animal organs.

Later investigations (5, 9, 14, 12) have shown that the nuclei of human liver cells also may be divided into classes according to nuclear volume. Furthermore it has been shown by microspectrophotometry that the DNA contents of hepatic nuclei belonging to neighbouring classes differ by a factor of 2 (11, 21, 5).

Most determinations of hepatic nuclear volumes have been performed on autopsy material, and it is uncertain if these observations reflect the in vivo volumes of hepatic nuclei. *Loeschke* (12) carried out karyometry on biopsies from 17 histologically normal livers. The biopsies were taken from patients that were suspected of suffering from liver disease, but the nature and degree of disease, if any, were not stated.

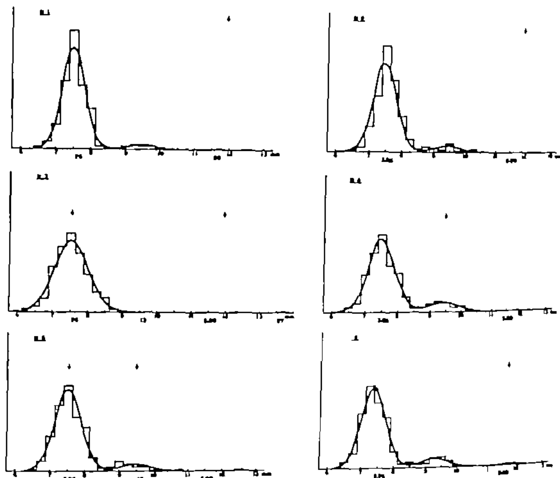


Fig 1 a.

Fig 1 a and 1 b Observed (histogram) and estimated (curve) frequency distribution of nuclear radii in ten patients with normal liver histology (N 1 – N 10) and two patients with acromegaly (A 1 + A 2) The abscissa gives the nuclear radii at 2300 × magnification and in μ. The arrows indicate mean nuclear radii of di tetra- and octaploid nuclei.

TABLE 1 *Age Sex and Diagnoses of the Patients*

Patient No.	Age (yr)	Sex	Diagnosis
N 1	22	F	Lupus erythematosus disseminatus
N 2	30	F	Familial intrahepatic fibrosis. *Not found*
N 3	36	F	Ventral hernia
N 4	50	F	Tumor of the breast
N 5	57	F	Collagen disease. Not classified
N 6	58	F	Chronic bronchitis
N 7	19	M	Pilonidal cyst
N 8	50	M	Anal fistula
N 9	53	M	Rheumatoid arthritis
N 10	62	M	Hemorrhoids
A 1	30	M	Acromegaly
A 2	34	M	Acromegaly

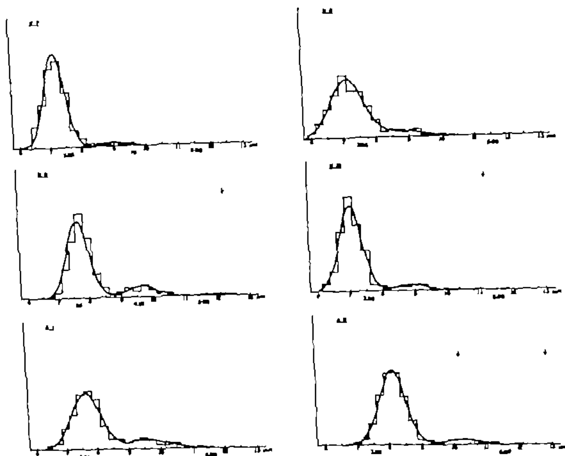


Fig 1 b

The purpose of the present work is to measure the nuclear size, to quantify the variation in size, and to estimate the prevalence of polyploid and binuclear liver cells in liver biopsies from patients with normal livers, as judged from clinical findings, routine laboratory tests, and liver histology

MATERIAL

The material is comprised of liver biopsies from twelve patients. The age, sex and clinical diagnosis of these patients are given in Table 1 Five of the patients (Nos. N 3 N 4 N 7 N 8, and N 10) are from study of the effect of anaesthesia on liver histology (2) The biopsies from these patients were taken before surgery In four patients (Nos. N 1 N 5, N 6, and N 9) the liver biopsy was performed for evaluation of suspected collagen disease One patient (No. N 2) was examined to exclude intrahepatic fibrosis Two patients with active acromegaly (A 1 and A 2) were studied to exclude other causes of heptomegaly Except for patient No. N 6, who underwent uncomplicated cholecystectomy 2 years previously no history indicative of earlier liver disease was elicited.

The only patient who admitted to regular alcohol intake was No. N 7 who drank several bottles of beer daily Drug intake was reported for No. N 1 (prednisolone + chloroquine) No. N 5 (indomethacin) and Nos. N 6 and 9 (salicylates) In the patients from the anaesthesia study serum bilirubin, serum aspartate aminotransferase and serum lactate dehydrogenase were normal. In the other patients liver tests also included prothrombin time, serum albumin, alkaline phosphatase, and bromsulphalein retention which all were within the normal range.

METHODS

The biopsy was performed on the fasting patient between 8 and 9 a.m. with a Menghini needle

(diameter 1.6 mm) The liver tissue was immediately fixed in 4 per cent aqueous formaldehyde buffered with phosphate to pH 7 and kept in the fixating medium at room temperature until approximately 2 or 3 p.m. when it underwent routine histological processing with dehydration in alcohol and embedding in paraffin. The section thickness was estimated to be between 4 and 8 microns (see page 5) but was not measured. Liver cell nuclear size was measured by the projecting method using a Reichert microscope (Zetopan) with oil immersion objective (100/1.25) and a 5 × plan ocular inserted in the photo-tube. The measuring field was projected onto a wooden plate covered with paper The final magnification was 2300 × and the measuring field approximately 40 cm in diameter

Liver cells in the periphery of the tissue cylinder corresponding to approximately three cell layers were not used for the measurements. Due to the small size of some of the biopsies, the measuring fields could not be selected at random, but the biopsies were scanned meandrously in non-overlapping areas. Only nuclei with a clearly and sharply outlined nuclear membrane were measured. The shortest and longest diameter of the nucleus was marked and later measured with a millimetre rule to the nearest mm. The mean of the two diameters was used for calculation of the length of nuclear radii. 500 mononuclear liver cell nuclei from each biopsy were measured, except in two cases, in which 250 and 300 nuclei were studied. All binuclear liver cells falling within the measuring field were counted, but no attempt was made to estimate the real frequency of binuclear cells (17)

Statistical Methods

The statistical methods used have been described in a previous paper (10) A parametric model for the distribution of the length of nuclear radii, the χ-distributions, makes the distribution of the size of the nuclear radii mathematically treatable This class of distributions describes the observations well (see Fig. 1) The requirement that the membrane should be clearly distinguishable was formalised in the model by counting sectioned nuclei only if the angle of the microtome with the nucleus was larger than some fixed angle (φ) It turned out in the three biopsies considered in the earlier paper that φ could be considered to be equal to 90° In other words, only nuclei with the equatorial plane in the section were counted. In the analysis it was assumed that the diploid, tetraploid, and octaploid nuclei radii follow χ-distributions with expectations increasing by a factor of 2⅓ from one ploidy class to the next and with an identical coefficient of variation in the three classes.

Comparisons of different biopsies were performed by likelihood ratio tests.

RESULTS

Studies on the Methodology

The section thickness A consequence of the assumption of $\varphi = 90$ is that the section thickness of the liver biopsy does not enter into the procedure for the estimation of the size of the nuclear radii. The adoption of this assumption is, however based on a definite presupposed section thickness. An analysis, not included in the previous paper (10) shows that for the three biopsies considered identical estimates result whether the section thickness is presumed to be 4, 6.5, or 8 μm, and that the overall fit was best for 6.5 μm. For the present calculations the section thickness was not measured, but a section thickness of 6.5 μm, which seems reasonable for routinely processed histological specimens, was used.

Precision of the method The precision of the projection method used was evaluated by moving the same nucleus into 30 different positions in the measuring field, and as usual marking the shortest and longest diameters on the paper The diameters were measured by a person not knowing that they were from the same nucleus. Tab. 2 shows that all measurements fall at two neighbouring points of the measuring scale giving a high degree of reproducibility

The effect of *optical distortion* from the center to the periphery of the measuring field (13) was evaluated by measuring the same nucleus in ten different positions in the central field and twenty different positions in the peripheral field. The diameters were measured with a vernier The results are presented in Tab. 3 which shows that no significant optical distortion was found. Thus, measurements can be made on a whole microscopic field at a time without loss of accuracy of measurement.

The *precision and reproducibility* of the measurements were evaluated by remeasuring 500 nuclei one month later The results are

TABLE 2. *The Precision of the Measuring Technique*

		Times recorded
Shortest diameter (mm)	16	4
Shortest diameter	17	26
Longest diameter	17	1
Longest diameter	18	29
Average of shortest and longest diameter	17	5
	17.5	25

Evaluated by moving the same nucleus to 30 different positions in the measuring field and marking the longest and shortest diameters on paper (magnification × 2300). The diameters were later measured to the nearest mm by a person not knowing it was the same nucleus.

given in Tab. 4 which show that the two series yield the same results (likelihood ratio test $\chi^2 = 0.44$ with 4 d.f.)

The question of *sampling error* was investigated by measuring a total of 2000 nuclei from the same biopsy divided into samples of 500 each. Two of the samples (1 and 2) were from one end of the biopsy and the other two samples (3 and 4) from the other end. The results are presented in Tab. 5. The comparison was performed in two steps. First, the lengths of the radii of nuclei from the same location in the biopsy were compared. The test of 1 = 2 gives $\chi^2 = 5.84$ d.f. = 4 which is not significant, whereas 3 = 4 gives $\chi^2 = 37.0$ d.f. = 4 which is highly significant (P<0.0005). Secondly an overall test that 1 = 2 = 3 = 4 gives $\chi^2 = 78.2$ with 12 d.f. which also is highly significant (P<0.0005).

Fixation. The length of time during which the liver tissue is fixed is known to influence the nuclear volume (16). To investigate the effect of fixation times 24 and 48 hours longer than usual, two liver biopsies were divided. One half of each biopsy was fixed for the normal 6 to 8 hours while the other half of one biopsy was fixed for an additional 24 hours, and the other half of the second biopsy was fixed for an additional 48 hours. As seen in Tab. 6 the nuclear volume tends to increase with increasing length of fixation. Therefore only biopsies with approximately the same fixation time were used.

TABLE 3. *Estimation of Optical Distortion*

	Central field (= 10)		Peripheral field (n = 20)	
Average of shortest diameter	16.72	0.18	16.79	0.20
Average of longest diameter	17.80	0.18	17.79	0.19
Average of both diameters	17.26	0.13	17.29	0.15

e same nucleus was measured in different positions in the central field and in the peripheral field the projected area. The diameters were measured with a vernier. Mean (mm) ± SD

TABLE 4. *The Reproducibility of Measurements of the Same 500 Nuclei*

	Mean radius of diploid nuclei (μm)	SD	Frequency of diploid nuclei	tetrapl. nuclei	octapl. nuclei
t measurement	3.07	0.15	90.90	8.40	0.70
od measurement	3.07	0.15	90.95	8.60	0.45

The shortest and longest diameters were marked on paper. Measurements on the paper were performed with an interval of one month. (Estimated mean nuclear radius of diploid nuclei and frequencies, in percentages of di-, tetra- and octaploid nuclei)

(diameter 1.6 mm) The liver tissue was immediately fixed in 4 per cent aqueous formaldehyde, buffered with phosphate to pH 7 and kept in the fixating medium at room temperature until approximately 2 or 3 p.m. when it underwent routine histological processing with dehydration in alcohol and embedding in paraffin. The section thickness was estimated to be between 4 and 8 microns (see page 5) but was not measured. Liver cell nuclear size was measured by the projecting method, using a Reichert microscope (Zetopan) with oil immersion objective (100/1.25) and a 5 × plan ocular inserted in the photo-tube. The measuring field was projected onto a wooden plate covered with paper The final magnification was 2300 × and the measuring field approximately 40 cm in diameter

Liver cells in the periphery of the tissue cylinder corresponding to approximately three cell layers were not used for the measurements. Due to the small size of some of the biopsies, the measuring fields could not be selected at random, but the biopsies were scanned meandrously in non-overlapping areas. Only nuclei with a clearly and sharply outlined nuclear membrane were measured. The shortest and longest diameter of the nucleus was marked and later measured with a millimetre rule to the nearest mm. The mean of the two diameters was used for calculation of the length of nuclear radii. 500 mononuclear liver cell nuclei from each biopsy were measured except in two cases, in which 250 and 300 nuclei were studied. All binuclear liver cells falling within the measuring field were counted, but no attempt was made to estimate the real frequency of binuclear cells (17)

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RESULTS

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The precision and reproducibility of the measurements were evaluated by remeasuring 500 nuclei one month later The results are

TABLE 7 *Distribution of the Observed Frequency of Liver Cell Nuclear Radii at 2300 Times Magnification in Ten Patients (N 1–N 10) with Normal Liver Function and Liver Histology and Two Patients with Active Acromegaly (A 1 + A 2)*

Radius (mm)	N 1	N 2	N 3	N 4	N 5	N 6	N 7	N 8	N 9	N 10	A 1	A 2
6.00			1					7		1		
6.25			5					21		11		
6.50	2	2	8	7	1	9	52	43		30	6	
6.75	6	7	19	15	7	22	68	64	1	90	9	1
7.00	21	37	63	55	25	78	121	94	9	144	33	3
7.25	58	81	94	87	48	121	133	70	44	101	53	8
7.50 (= 3.26 μ)	101	153	113	113	62	96	84	69	87	62	85	32
7.75	54	104	84	68	59	75	26	47	131	10	91	77
8.00	35	52	54	57	92	24	13	26	95	7	78	109
8.25	3	18	29	22	10	6	6	8	37	4	56	110
8.50	1	4	21	6	2	7	4	11	15	6	14	74
8.75	1	8	4	8	3	5	2	11	6	6	15	37
9.00	3	6		1	7	6	6	9	8	8	9	7
9.25	4	3		16	5	14	3	11	14	9	13	6
9.50 (= 4.13 μ)	3	12		15	3	15	4	2	9	4	17	3
9.75	3	2	1	7	3	10		1	18	1	13	5
10.00	2	2		9	1	2		3	11	2	7	7
10.25	1	4		3		2		2	6	2	5	8
10.50		1		1		1			5		8	7
10.75					1					1	4	1
11.00						1			1		1	1
11.25				1		1					2	3
11.50 (= 5.00 μ)						1					1	
11.75	1					2			2	1		
12.00		1		1		1		1	2			1
12.25		1	1	1		1						
12.50	1	1			1				1			
12.75												
13.00		1										
13.25												
13.50 (= 5.87 μ)												
13.75			1									
14.00												
Total number	500	500	500	500	250	500	500	500	500	500	500	500

DISCUSSION

In the statistical evaluation of the measured nuclear radii used in the present investigation it is assumed that these form approximately normal distributions, and that the mean nuclear volume is doubled from one nuclear class to the next, with identical coefficients of variation. The data in the present material fit well with the concept that the nuclei of different ploidy classes form nuclear classes and that the nuclear radii form normal distributions.

The observations are not in agreement with the conclusion of *Elias* & *Sherrick* (4) made from recalculation of the karyometric data of *Clara* (3) that tetraploid nuclei are absent in the human liver and that di- octa- and dodecaploid prevail. The present findings are supported by microspectrophotometric investigations of the DNA content of liver cell nuclei (11 21 5 18). No support of the existence of "Zwischenklassen" (7) has been found, but there is some overlapping in the size distributions of nuclear radii of the different ploidy classes.

TABLE 8. *Estimated Mean Nuclear Radius of Diploid Nuclei with Standard Deviation and Coefficient of Variation (CV)*

Patient No.	Age (yr)	Sex	Diploid nuclei mean radius (μ)	SD	CV	Estimated frequencies* of diploid nuclei	tetraploid nuclei	octaploid nuclei	Observed freqs. binuclear liver cells§
N 1	22	F	3.27	0.14	4.3	93.6	5.7	0.7	50
N 2	30	F	3.28	0 15	4.6	91.8	7 4	0.8	97
N 3	36	F	3.27	0.21	6.4	99 4	0.3	0.3	40
N 4	50	F	3.26	0 17	5.2	85.2	14.2	0.6	68
N 5	57	F	3.27	0.17	5.2	90 4	9.2	0.4	87
N 6	58	F	3.20	0.16	5.0	86 4	12.2	1 4	63
N 7	19	M	3 11	0 16	5 1	95.6	4 4	0	110
N 8	50	M	3.11	0.22	7 1	89.0	10.8	0.2	119
N 9	53	M	3.37	0.15	4.[illegible]	84.3	14 7	1 0	116
N 10	62	M	3.07	0.15	4.9	90.9	8.4	0.7	105
A 1	50	M	3.34	0.21	6.3	82.9	17 1	0	115
A 2	54	M	3.53	0.18	5.1	92.5	7.5	0	82

Estimated frequencies of di- tetra- and octaploid nuclei and observed frequency of binuclear liver cells.
* Percentages.

$$§\ \left(\frac{\text{number of binuclear cells}}{\text{number of binuclear + mononucl. cells}} \times 100\right)$$

Swartz (21) has shown that polyploid liver cell nuclei are less common in infant livers, that tetraploid nuclei develop around puberty and that octaploid nuclei are found from approximately the age of twenty In the present adult material the frequency of polyploid nuclei increased with age, and this becomes even more evident when the results from *Loeschke* (12) are included as shown in Fig 2.

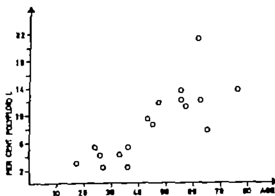


Fig 2 The correlation between age (yr) and frequency of polyploid liver cell nuclei. Closed circles indicate results from present study open circles from *Loeschke* (12)

An increased frequency of polyploid nuclei is also seen during regeneration in human livers (1 19)

Helweg-Larsen (6) has shown that in hereditary pituitary dwarf mice only diploid nuclei exist, and administration of growth hormone restores the normal nuclear classes including tetra and octaploid nuclei. Growth hormone accordingly induces the formation of polyploid nuclei. Both patients in the present material with acromegaly had hepatomegaly Only one of them (A 1) had an increased frequency of tetraploid nuclei compared with the controls. The size of the diploid nuclei in these patients was large and in one (No. A 2) significantly larger than in the controls. *Tager* (22) found normal liver cell nuclear size and no increase in the frequency of polyploid nuclei in a 56 year old man who died after brain surgery for acromegaly but liver lobules were found to be enlarged to about 150 per cent of normal. Thus several different mechanisms may be responsible for the hepatomegaly found in acromegaly

In the present material a significantly

higher frequency of binuclear liver cells was found in males than in females. This was also found in a material of 3 males and 7 males with acute virus hepatitis (19). However the values of binuclear cells in *Loeschke's* paper (12) do not indicate any difference between males and females.

From animal experiments *Nadal* (15) concluded that binuclear diploid liver cells are the origins of clones of polyploid mononuclear cells. Thus binuclear cells with diploid nuclei give rise to tetraploid nuclei after a simultaneous prophase in both nuclei and the formation of only one spindle. The following division results in the formation of two tetraploid nuclei. The presence of binuclear cells, however does not inevitably lead to the formation of polyploid nuclei. This can be deduced from *Helweg-Larsen's* observation (6) that the hereditary pituitary dwarf mice have binuclear diploid liver cells, but no polyploid nuclei. The formation of polyploid nuclei that occurred when growth hormone was administered might have been due to transition of mononuclear diploid cells to polyploid cells. The possibility that polyploid nuclei may be formed both from binuclear cells and from mononuclear cells thus cannot be excluded.

Helweg-Larsen (6) found that the individual nuclei in binuclear cells tend to be smaller than mononuclear nuclei of the same class. This was not the case in the present investigation where the nuclei of binuclear cells are of the same size as that of mononuclear cells. Previous investigations on human livers (3 14 12) are in agreement with this.

The variance in nuclear size in the normal liver as judged from histological specimens is small, but the nuclear volume may be influenced by nutrition, toxic and non-toxic drugs, hormones, and regeneration (20). A great variation in nuclear size in human livers is also seen in pathological conditions (12 7 19). In the present study the variation in nuclear size in patients who received drugs did not differ from that in the other patients.

The present investigation also demonstrates a biological variation in nuclear size and frequency of polyploid nuclei within the same biopsy and this variation must be taken into account when karyometric data are interpreted.

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KARYOMETRY OF LIVER BIOPSIES IN VIRUS HEPATITIS

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Liver biopsies from ten patients with clinically mild or moderate acute virus hepatitis, taken in the acute phase of the disease and in the recovery phase or later were assessed for the size of liver cell nuclei and the number of binuclear nuclei. A parametric model of the distributions of the nuclear radii was used to estimate the mean nuclear radius of diploid nuclei and the frequencies of di-, tetra- and octaploid nuclei. During the acute phase of virus hepatitis the liver cell nuclei were often larger, with greater variation in size, than in the recovery phase. This pleomorphism was not only due to pyknotic and necrotic nuclei as these were not measured. It is assumed that the enlargement of the nuclei was due to increased metabolic activity of the nuclei. The frequencies of polyploid nuclei and binuclear liver cells were higher in the first biopsies, probably reflecting regenerative activity. No correlation was found between the severity of the disease as judged by liver histology or routine liver tests and the degree of nuclear changes with respect to size and frequency of polyploid or binuclear liver cells.

Key words: Liver biopsies; karyometry; virus hepatitis.

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The histological picture of the liver in virus hepatitis in man is characterized by, among other things, a pleomorphism of the liver cell nuclei, as already observed by *Roholm & Iversen* in 1939 (10). This has been confirmed by karyometric investigations (5, 3, 2), but a karyometric quantification of the nuclear changes has been difficult due to the lack of appropriate statistical methods by which the different ploidy classes of the liver cell nuclei can be separated and quantified.

The purpose of the present work is to quantify the hepatic nuclear variation by karyometry of liver biopsies from patients with acute virus hepatitis, using a statistical method which has been found to describe the observed nuclear variations well (4). The nuclear sizes are correlated with a histological grading of the severity of the disease and with the results of some routine liver tests.

Besides the changes in nuclear size, changes in the frequencies of polyploid nuclei and binuclear liver cells, probably indicating regenerative processes, are evaluated.

TABLE 1 *Age Sex Type of Virus Hepatitis and Interval from the Onset of Symptoms to the First Liver Biopsy and between Following Biopsies. Results f Liver Tests at the Time of Biopsy and the Histological Grading of the First Biopsy*

Pt. No.	Age (yr) Sex	Type A or type B hepatitis	Interval from start of symptoms (0) to first biopsy (1) and between successive biopsies	Bilirubin (<1.0 mg/100 ml)	Prothrombin (85–115 per cent)	Albumin (>4.4 g/100 ml)	Alanine aminotransferases (<1.8 µmol/h/ml)	Histological ranking (10 = severest case)
H 1	45 F	B	0–1 21 d	2.9	85	3.60	52.0	3
			1–2.: 510 d	0.6	100	4.62	0.6	
H 2	55 F	B	0–1 36 d	2.8	81	4.56	50.0	10
			1–2. 21 d	0.5	76	4 65	2.5	
			2–3 180 d	0.4	100	5 10	0 7	
H 3	70 F	A	0–1 20 d	5.5	55	3.30	6.9	8
			1–2 26 d	1 1	54	3 44	3.9	
			2–3 ; 360 d	0.5	95	4 15	0.5	
H 4	19 M	A	0–1 19 d	2.6	65	4 05	5.5	4
			1–2. 11 d	1.5	160	4.05	1.5	
H 5	22 M	A	0–1 14 d	5.5	110	3.95	5.3	2
			1–2. 12 d	0.9	100	3.84	1.8	
H 6	23 M	A	0–1 16 d	9.0	53	4 40	5.2	1
			1–2. 35 d	1.2	105	3.63	4 0	
H 7	23 M	A	0–1.: 8 d	8.6	57	4.00	95 4	9
			1–2.: 15 d	1 6	100	–	6.0	
H 8	53 M	A	0–1 : 23 d	10.9	90	3.39	5.3	6
			1–2,: 33 d	0.8	88	–	2.3	
H 9	41 M	A	0–1 : 10 d	4.6	59	2.55	54 2	5
			1–2. 18 d	0 6	87	4 42	4.2	
H 10	55 M	A or B	0–1 : 20 d	16.8	46	3 67	35 2	7
			1–2. 20 d	4.3	96	3 77	6.2	
			2–3. 80 d	0.3	95	4.31	0 9	
			3–4 300 d	0.5	112	4 60	0.4	

MATERIAL

The material is comprised of liver biopsies from ten patients with acute virus hepatitis, in whom two or more liver biopsies had been performed. Chronic liver disease had not developed in any of the patients. The diagnosis of virus hepatitis was based on clinical and epidemiological informations. HBAg determinations were not performed at the time the biopsies were collected. None of the patients was severely ill clinically. Two patients had three biopsies and one patient four. Karyometry was performed on all 24 biopsies.

The age and sex of the patients are given in Table 1 together with the assumed type of virus hepatitis, the interval between the first symptom and the first liver biopsy and between successive biopsies, and the routine liver tests performed at the time of biopsy.

METHODS

Liver biopsies were performed with a 1.6 mm Menghini needle in the fasting patient between 8 and 9 a.m. The processing of the tissue and measurement of nuclear diameters were performed as described in a previous paper (8). Only liver cell nuclei with a clearly distinguishable membrane were measured. Pyknotic nuclei with homogenously stained nuclear material and nuclei with a blurred membrane were not measured. Accordingly the frequency of these nuclei compared with the frequency of measured nuclei cannot be evaluated. 500 nuclei of mononuclear liver cells from each of 22 biopsies and 250 nuclei from each of two biopsies were measured. From the observed distributions the mean nuclear radii and frequencies of di-, tetra- and octaploid mononuclear nuclei were estimated by a parametric model (4).

Binuclear liver cells and mitoses of liver cells observed in the measuring field were counted.

The initial biopsies were evaluated histologically by a hepatologist and ranked from one to ten according to the degree of tissue damage, 1 and 2 being regarded as mild, and 8, 9 and 10 as severe cases. The severity was evaluated by the extent of parenchymal necroses and of inflammation in the portal tracts. In the biopsies ranked as 1 and 2 only few necroses and few inflammatory cells were found, whereas many necroses sometimes confluent, and heavy infiltration with inflammatory cells were found in the biopsies ranked as 8, 9 and 10. Subtotal liver cell necrosis was not seen.

The following liver tests were carried out. Serum bilirubin, prothrombin, serum albumin and serum alanine aminotransferase.

RESULTS

The distribution of the observed frequencies of the lengths of the nuclear radii is given in Table 2. In biopsy No. 3 from patient H 10 pale nuclei with condensation of chromatin along the nuclear membrane were frequent and were measured separately (3 b). In Fig. 1 a + 1 b the observed frequencies of the lengths of the nuclear radii are shown together with the estimated frequencies from the first biopsies of all patients.

The estimated mean nuclear radii of diploid nuclei are given in Table 3. For the material as a whole the liver cell nuclei were significantly larger ($t = 2.3$, $p < 0.05$) in the acute phase of the disease than in the recovery phase. The variance in size was significantly higher in the first biopsies. The nuclei were also significantly larger than those from a material of normal liver cell nuclei (8) ($t = 2.53$, $p < 0.05$).

The estimated frequencies of di-, tetra- and octaploid nuclei are given in Table 3. If the initial and second biopsies in all patients are compared, there was a significant ($t = 3.82$, $p < 0.01$) reduction in the frequency of polyploid nuclei. Thus the size of the nuclei within each ploidy class decreased during recovery and the overall nuclear size also decreased due to a reduced frequency of polyploid nuclei. The variance in nuclear size within a ploidy class was not correlated with the frequency of polyploid nuclei.

The observed frequency of binuclear liver cells

$$\left(\frac{\text{number of binuclear cells}}{\text{number of binuclear + mononuclear cells}}\right) \times 100$$

and liver cell mitoses are also given in Table 3. In the whole material there was a significant reduction in the number of binuclear cells from the first biopsy to the second one. The correlation between the decrease in binuclear cells and the decrease in polyploid mononuclear cells is significant ($p < 0.05$).

As found in normal livers (8) the frequency of binuclear cells was significantly higher in males. Mitoses were seen in seven

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TABLE 2. *Distribution of the Observed Frequency of Nuclear Rad*

Radius (mm)	H.1		H.2			H.3			H.4	
	1	2.	1	2.	3.	1	2.	3.	1	2.
5.75		1								
6.00		2				1				
6.25	1	15			1			2	11	
6.50	5	36		1	3	7	8	7	16	7
6.75	10	58			9	29	28	17	30	34
7.00	18	90			30	58	43	35	55	71
7.25	24	97		1	43	74	72	46	60	85
7.50 (= 3.26 μ)	43	81		2	51	87	72	57	83	121
7.75	61	48		2	46	82	75	42	62	63
8.00	65	27		13	29	60	64	21	74	51
8.25	62	22	1	26	14	33	43	5	31	16
8.50	60	7	3	47	11	19	29	5	22	16
8.75	57	8	11	67	6	6	10	2	12	3
9.00	30	2	10	73	3	3	11	2	5	3
9.25	17	2	21	73	1	3	7	2	7	3
9.50 (= 4.13 μ)	15	1	40	65		4	6	3	5	1
9.75	11	2	42	60		3	5	2	6	3
10.00	8	1	34	32		4	4		4	3
10.25	6		36	21		5	4	2	1	1
10.50	7		57	4	1	3	6		2	1
10.75	2		46	6		1			1	
11.00			43			1			3	
11.25	5		39	1		1	1			
11.50 (= 5.00 μ)	5		24			1	1		2	
11.75	2		15			3	1		2	
12.00			10	2		1	1			1
12.25	2		10	1			2			
12.50			3	1			1			
12.75	1		2			2				
13.00	2		3	1		1				
13.25			1			2				
13.50 (= 5.87 μ)						1				
13.75						1	1			
14.00			4							
14.25	1		1	1			2			
14.50			1				1			
14.75			1							
15.00										
15.25										
15.50 (= 6.74 μ)			1							
15.75										
16.00							1			
16.25										
16.50										
16.75										
17.00										
17.25										
17.50 (= 7.61 μ)										
17.75										
18.00										
18.25							1			
18.50										
18.75			1							
Total number	500	500	500	500	250	500	500	250	500	500

Interval between biopsies, see Tab. 1 Patient No. H.1 H.2 etc. as in Tab. 1

at 2300 Times Magnification of Ten Patients with Viral Hepatitis

H.5		H.6		H.7		H.8		H.9		H.10				
1	2.	1	2.	1	2.	1	2	1	...	1	2.	3a	3b	4
1		4					1			1	2			
		8	1		1	1	9	1		2	12		1	
5	9	42	4		3	9	20		2	5	27	17	1	15
9	28	53	9	2	14	8	51	2	7	9	48	32	4	38
21	30	93	34	2	35	28	79	3	15	35	85	67	7	71
44	97	89	61	12	51	72	122	7	28	36	83	85	30	79
44	98	79	76	25	87	87	113	24	37	50	81	126	63	109
51	78	58	99	51	73	91	55	44	51	44	68	79	78	68
43	47	23	79	68	78	79	22	58	68	59	34	49	90	44
55	26	9	56	69	46	47	9	67	63	25	19	28	77	29
43	17	14	31	60	34	1	9	69	61	42	13	6	62	17
39	4	10	9	47	22	23	3	57	41	32	5	3	52	7
30	7	7	6	33	11	8	2	49	33	28	3	2	14	6
23	9	7	7	25	5	4		28	28	32	3	2	5	4
16	5	6	1	18	7	4	1	18	12	16	4	1	1	3
14	7	5	5	7	9	1	3	13	10	16	2		4	1
10	3	4	6	14	6	3	1	10	10	9	1		1	1
7	3	2	4	14	8	7		4	4	11		2	3	
5	4	2	3	12	2	1		4	1	7	1		2	3
7	3	2	2	10	1	4		9	4	4		1	1	
5	3	1		6	1			7	2	7	2		3	
7	1			6				1		3			1	
2	1			8	2	1		3	1	3	1			1
3		1	1	1				4	1	2	1			1
4			2	2				3		2				
1		1	1	1				4	1	6	1			1
			1	1	1			3		4				
1				1				1		2				
2						1					2			
								1		3	1			
								2		1				
										1	1			
				1	1									
								1		1				
								1						
								1						
1										1				
										1				
								1						
500	500	500	500	500	500	500	500	500	500	500	500	500	500	500

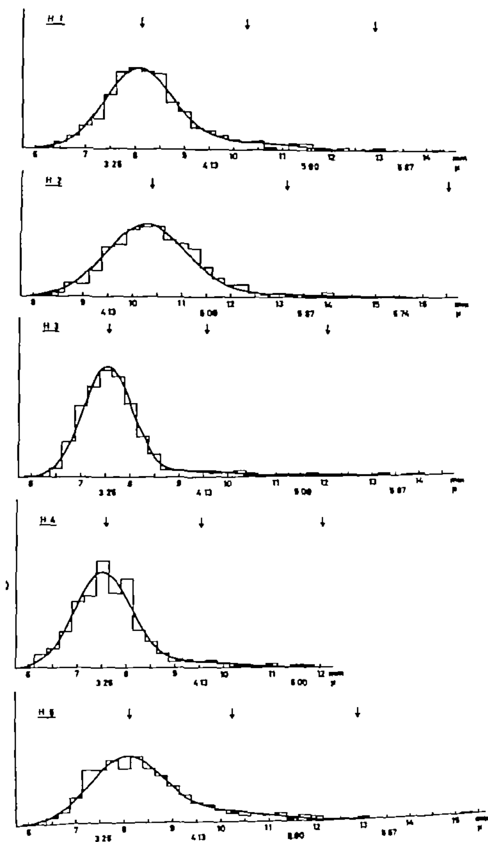


Fig 1a.

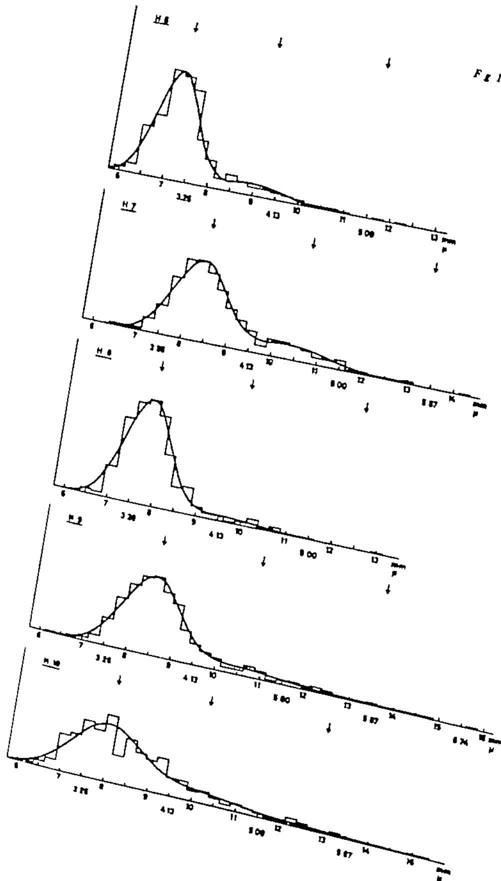


Fig 1 b
H 6
H 7
H 8
H 9
H 10
mm
μ

of the initial biopsies, but in only one (H 9) of the second biopsies.

No correlation was found between the mean nuclear radius of diploid nuclei and the interval between the first symptom due to hepatitis and the first liver biopsy nor were any significant correlations found between nuclear size or variance in size and the different liver tests or the histological ranking

DISCUSSION

The present results demonstrate that during the acute phase of virus hepatitis the hepatic nuclei often are larger and exhibit greater variation in size than the nuclei in normal livers, even though the necrotic, homogeneously stained nuclei and nuclei with blurred membranes were not measured. The statistical method used (4) permitted an estimation of the nuclear size distributions and frequencies of the different ploidy classes. As found in normal livers (8) the statistical evaluation of the observed frequency distributions of nuclear radii is in agreement with the concept that the different nuclear ploidy classes form approximately normal distributions. In Fig 2 the estimated normal distributions of nuclear radii of di and tetraploid nuclei from patient No. H 7 is shown. The figure demonstrates the overlapping in size distribution between di and tetraploid nuclei. The nuclear volume of diploid nuclei may be more than doubled during the acute phase of the disease as shown in Fig 3 where the estimated size distribution of diploid nuclei from three successive biopsies from the same patient (H 2) is presented. The mean nuclear volume of diploid nuclei is 398 μ^3 in the first, and 153 μ^3 in the third biopsy taken approximately six months later The nuclear DNA content was not measured, but cytophotometric investigations have shown that diploid nuclei always constitute the majority of human liver cells (9) The frequencies of polyploid nuclei and binuclear liver cells are higher in the initial liver biopsies, compared with later biopsies from the same patients. As mitoses were seen predominantly in the initial biopsies, the higher frequencies of polyploid and binuclear liver cells probably reflect regenerative processes. While these changes indicate DNA-synthesis, the main cause of the enlargement of the nuclei may be increased RNA and protein synthesis. This hypothesis is supported by microspectrophotometric and interferometric investigations (7) in which positive correlations between nuclear size and nuclear protein, and nuclear size and dry weight within ploidy classes are found, whereas the correlation between nuclear size and DNA content is poor Amino acid incorporation studies in liver biopsies from patients with virus hepatitis have shown an increased protein synthesis rate (10) and electronmicroscopic changes in the nuclei are also consistent with an increased metabolic activity (2)

As pointed out in a previous paper (8) only mononuclear liver cell nuclei with the

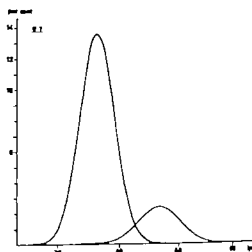


Fig 2 Estimated size distribution of di- and tetraploid liver cell nuclei from patient H 7 showing normal distributions with overlap areas.

Fig 1 a and b Observed (histogram) and estimated (curve) frequency distribution of nuclear radii in ten patients with acute virus hepatitis (first biopsies) The abscissa gives the nuclear radii at 2300 × magnification and in μ The arrows indicate mean nuclear radii of di- tetra- and octaploid nuclei. (The abscissa in patient H 2 begins at 2 mm higher level than the others)

TABLE 3. *Estimated Mean Nuclear Radii of Diploid Nuclei, Estimated Frequencies of Di-, Tetra- and Octaploid Nuclei and Observed Frequencies of Binuclear Cells and Liver Cell Mitoses*

Pt. No.	Biopsy No.	$\bar{x}$ nuclear radius (μ) ± SD		Estimated frequencies (per cent) dipl.	tetrapl.	octapl.	Observed frequencies (per cent) binucl.	mitoses
H 1	1	3.53	0.30	88.7	10.1	1.2	4.7	1.4
	2	3.15	0.23	96.8	3.2	0	5.1	0
H 2	1	4.53	0.36	96.2	3.4	0.4	5.0	0
	2	4.00	0.27	98.5	1.3	0.2	1.6	0
	3	3.30	0.22	98.8	1.2	0	3.8	0
3	1	3.29	0.21	90.9	6.3	2.8	4.0	1.2
	2	3.34	0.27	90.5	6.8	2.7	3.0	0
	3	3.22	0.19	94.4	5.6	0	7.4	0
4	1	3.28	0.25	91.8	7.2	1.0	12.0	0
	2	3.31	0.19	92.6	7.2	0.2	15.0	0
H 5	1	3.52	0.33	83.4	15.4	1.2	15.5	0
	2	3.27	0.20	89.7	9.6	0.7	14.4	0
H 6	1	3.12	0.20	87.1	11.8	1.1	12.9	0.2
	2	3.37	0.21	92.4	6.2	1.4	10.7	0
H 7	1	3.61	0.26	80.9	18.0	1.1	13.8	1.6
		3.37	0.23	89.5	9.9	0.6	11.0	0
H 8	1	3.36	0.23	94.0	5.7	0.3	6.5	0.2
	2	3.17	0.19	97.5	2.5	0	4.5	0
H 9	1	3.67	0.28	87.5	9.8	2.7	10.9	0.2
	2	3.56	0.30	94.7	5.3	0	7.2	0.4
H 10	1	3.48	0.33	78.0	17.5	4.5	8.0	0.2
	2	3.19	0.23	94.3	3.6	2.1	3.0	0
	3a	3.24	0.20	98.2	1.8	0	2.5	0
	3b	3.50	0.22	96.8	3.2	0	3.5	0
	4	3.24	0.22	94.7	4.6	0.7	3.8	0

equatorial plane in the section were measured. This is not the case when binuclear liver cells are counted, and due to the fact that one of the two nuclei may be outside the section, the frequency of binuclear cells is underestimated in sections (6, 1). In the present work no attempt to calculate the real frequency of binuclear cells has been made, and this frequency is probably better estimated in liver cell suspensions (12).

The nuclear changes could not be correlated either to the histological grading of the severity of the disease or to the results of the liver function tests used. The lack of correlation between the histological estimation of the severity of the disease on one hand, the results of commonly used liver tests and the clinical condition of the patient is, however well known.

Few reports exist on karyometry of liver biopsies from patients with virus hepatitis. *Loeschke* (5) investigated liver biopsies from four patients, of whom two had a liver biopsy performed twice. In the initial biopsies of all patients the frequency distributions of nuclear areas were described as broad peaks each with a maximum corresponding to greater nuclear areas than seen in normal biopsies, and without a peak corresponding to tetraploid nuclei. An increased number of tetraploid nuclei was not mentioned as a possible explanation, but the frequency distribu-

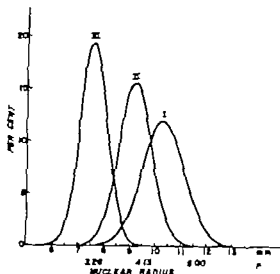


Fig 3 Estimated size distribution of diploid nuclei only in three successive biopsies from the same patient (H 2) I = first biopsy

tion curves of nuclear areas presented in *Loeschke's* study may well have been due to the increased frequency of polyploid nuclei with greater overlap areas, as was shown to be the case in the present investigation. In Loeschke's work a higher number of binuclear cells was also observed in the acute phase of the disease in the patients where two biopsies were performed. It is more difficult to compare our results with those of *Craciun & Tasca* (3) who reported measurements of nuclear volumes and internuclear distances in one normal liver and in the livers of two patients with virus hepatitis, in whom repeated biopsies were performed. They describe several peaks in the size distribution of the hepatic nuclei, and a separation in ploidy classes is difficult to see. Changes in DNA content were assumed to explain these findings, but this is not in accordance with spectrophotometric investigations (8). However it appears from their measurements, that they also *found the hepatocyte nuclei to be larger* in the acute phase of the disease.

The possible prognostic value of karyometry *cannot* be *evaluated on the basis of our* findings, as all of our patients recovered. However considering the great interpatient *variations* observed and the variation in size and frequency of polyploid nuclei within the same biopsy (4) prognostication on the basis of karyometry seems questionable.

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Acta path. microbiol. scand. Sect. A, 83: 487–492, 1975

BACTERIAL ANTIGEN AND ACID PHOSPHATASE IN MACROPHAGES IN EXPERIMENTAL PYELONEPHRITIS

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Thomsen, O. F. Bacterial antigen and acid phosphatase in macrophages in experimental pyelonephritis. Acta path. microbiol. scand. Sect. A, 83: 487–492, 1975.

Experimental retrograde *E. coli* pyelonephritis was produced in rats. The study covered the period from 6–24 hours up to 6 months. Macrophages in the renal tissue were studied using immunofluorescence staining for bacterial *E. coli* antigen and histochemical staining for acid phosphatase. A comparison of sections stained according to the two methods showed that antigen-containing macrophages in nearly all cases yielded a positive reaction for acid phosphatase. On the other hand, in several kidneys acid phosphatase-positive macrophages occurred which in consecutive sections studied by immunofluorescence did not contain antigen. The possibility of using staining for acid phosphatase as a screening method for the detection of active antigen-containing macrophages in human chronic pyelonephritis is discussed.

Key words: Pyelonephritis, experimental; macrophages; bacterial antigen; acid phosphatase.

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In many cases of chronic renal disease classified by current histological criteria (6) as chronic pyelonephritis, the aetiology is unknown, and it has been questioned whether microorganisms are at all responsible. In 1969 Aoki *et al.* (1) published a report on the demonstration by immunofluorescence of a common enterobacterial antigen (CA) in patients with clinically chronic pyelonephritis. However, other investigators were unable to confirm this (9), and studies of experimental pyelonephritis in rats have shown that CA persists for a relatively short time in the tissue, whereas specific bacterial 0-antigen can be demonstrated for a longer time (11, 12). A characteristic feature is the presence of bacterial antigen in macrophages, in which it can be demonstrated 2–3 days after the induction of the infection and onwards during the entire active inflammatory period (12).

In *human* chronic renal disease, a search for bacterial antigen by immunofluorescence would therefore have to include a wide panel of antisera against pathogenic strains, which would require a fairly extensive study. However, the examination would be simplified if kidneys could be selected which were particularly liable to contain bacterial antigen, i.e. kidneys containing active macrophages.

Macrophages can be demonstrated histochemically on the basis of their lysosomal content of, for example, acid phosphatase (5, 13). Several *in vitro* investigations have

shown a relationship between enzymatic and phagocytic functions of the macrophages (3 4 10) whereas this problem has been investigated only to a small extent *in vivo* employing tissue sections (7)

As a prerequisite for the use of staining for acid phosphatase as a screening method, it is necessary to make sure that bacterial antigen-containing cells will yield a positive reaction for acid phosphatase, as observed in tissue sections from pyelonephritic kidneys, and to find out whether a constant relationship exists between the content of bacterial antigen and acid phosphatase in the macrophages.

The aim of this study was to investigate this by observing the simultaneous occurrence of *E coli* antigen-containing cells and acid phosphatase positive cells in experimental pyelonephritis.

MATERIAL AND METHODS

Experimental retrograde pyelonephritis was produced in white female Wistar rats by injection of *E. coli* 04 U4/41 The animals were killed at different time intervals after the injection covering a period from 6–24 hours up to 6 months. Kidney tissue was quick-frozen, and cryostat-cut sections were stained for *E. coli* 04 antigen, for acid phosphatase and with haematoxylin-eosin and periodic-acid-Schiff (PAS) Staining for bacterial antigen was carried out by the indirect immunofluorescence technique. Details of the experimental procedures and immunofluorescence technique have been described in a previous paper (12) After fixation of cryostat-cut sections in formalin-$CaCl_2$ for 10 minutes at 4 C, staining for acid phosphatase was carried out using *Burstone* azo-dye (napthol AS-MX phosphate) method for staining of acid phosphatase in frozen sections (2) The slides were washed in distilled water for 10–20 seconds and then immersed into the substrate for 45 minutes. Then the sections were stained with Harris haematoxylin for 2½ minutes and mounted in Paragon.

RESULTS

The study included 74 rats with pyelonephritis of one or both kidneys. A total of 106 kidneys showing macroscopic evidence of acute or chronic pyelonephritis (i e. yellowish/white infiltrates or cortical contractions, respectively) were examined. The general gross and light-microscopic picture of the experimental pyelonephritis in rats, together with the localization and persistence of the bacterial antigen, as seen at the immunofluorescence examination, has been described in detail previously (12)

Immunofluorescence Macrophages containing phagocytized bacterial antigen appeared with a bright green cytoplasmic fluorescence mostly covering the entire cytoplasm, but sometimes appearing as distinct phagosomes. Numerous macrophages occurred from the 2nd–3rd day of inflammation. They could be grouped according to three characteristic localizations in the *cortex* (in 71 of 106 kidneys) at the *fornix* (in 49 kidneys) and *around abscesses* (in 13 kidneys) In the cortex, the number of macrophages decreased during the 2nd month, the cytoplasmic fluorescence gradually assuming the form of smaller clumps. The latest time they were observed was in the 4th month of inflammation. At the fornix, antigen-containing macrophages persisted for a longer period. Thus, this investigation includes a few animals with weakly fluorescent macrophages in the fornical region after 6 months, whereas no antigen-containing cells could be demonstrated in the contracted cortical areas Around abscesses, fluorescent macrophages were *not seen* later than the 2nd month. Fluorescent macrophages were not present in renal tissue without inflammatory changes.

In PAS-stained sections, the cells *around* abscesses, and in many cases, the fornical cells, were PAS-positive, whereas this was rarely the case with the cortical macrophages.

A positive reaction for *acid phosphatase* is present in normal mammalian kidney tissue (14) mainly in the epithelial cells of the proximal convoluted tubules in the form of fine, uniformly distributed cytoplasmic granules, representing lysosomes. These are stained red with the azo-dye technique, yielding a distinct contrast to the bluish-violet colour of the other tissue components. During the inflammation, also other cells occurred, isolated or in groups they were of an appearance

TABLE 1 *The Results of Comparison between Immunofluorescent Staining and Staining for Acid Phosphatase in 106 Rat Kidneys with Pyelonephritis*

Period	No. of animals with pyelonephr.	No. of kidneys with pyelonephr.	No. of kidneys with the same localization of antigen-containing macroph. and IAPh-cells			No. of kidneys with ag-containing macroph. and no IAPh-cells in the corresponding localization			No. of kidneys with IAPh-cells and no ag-containing macroph. in the corresponding localization		
			Cortex	Fornix	Periabsc.	Cortex	Fornix	Periabsc.	Cortex	Fornix	Periabsc.
6–24 hours	6	10	–	–	–	–	–	–	–	–	–
2–7 days	17	23	22	9	5	0	1	0	0	3	5
8–14 days	13	17	16	14	3	0	1	2	0	0	0
15–28 days	11	17	16	10	2	1	1	0	0	3	1
2nd month	9	14	12	8	1	0	0	0	2	3	0
3rd month	8	11	2	2	0	1	0	0	3	2	0
4th month	5	8	1	0	0	0	1	0	4	0	0
at 180 days	5	6	0	2	0	0	0	0	4	0	0
Total	74	106	69	45	11	2	4	2	15	11	6
Percentage			65.1	42.5	10.4	1.9	3.8	1.9	14.2	10.4	5.7

–: no macrophages demonstrable in the renal tissue (6–24 hours)

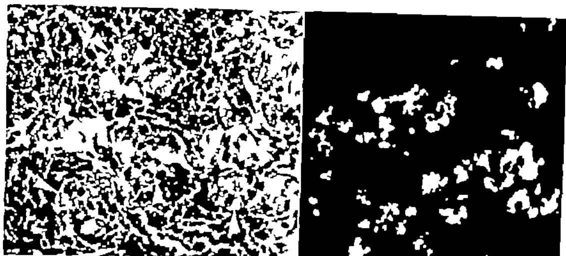


Fig 1 Left groups of fornical cells (arrows) with fine cytoplasmic granules in section stained for acid phosphatase. The cytoplasm of these cells was light red owing to the numerous small, uniformly distributed granules, yielding a contrast to the violet-bluish of the surrounding tissue. Right same group of cells in a consecutive section stained with antiserum against *E. coli* 04. The fluorescent cytoplasm indicates that the cells are macrophages with phagocytized bacterial antigen. × 500.

similar to that of macrophages and contained acid phosphatase-positive cytoplasmic granules of varying size from fine to coarse. Such cells were not found in non-inflamed renal tissue, and owing to their characteristic appearance and localization they could not be mistaken for the normally occurring interstitial medullary cells which have a content of various enzymes, e.g. acid phosphatase (8).

Numerous interstitial acid phosphatase positive cells (hereafter referred to as IAPh cells) occurred from the 2nd–3rd day with the same main localizations as the macrophages seen by immunofluorescence. In the *cortex* IAPh-cells were seen in 84 out of the 106 kidneys at first they were numerous, then occurring in decreasing numbers and gradually with fewer lysosomes. At the *fornix groups of IAPh-cells were seen in 56 kidneys*, from the 3rd month with sparse cytoplasmic granulation. *Around abscesses* IAPh-cells were seen in 17 kidneys, generally in large numbers. Around abscesses and often at the fornix, IAPh-cells generally contained smaller and more uniformly distributed lysosomes, than in the cortex.

Comparison between sections stained for bacterial antigen and for acid phosphatase

By comparing consecutive sections from the same kidney one of them stained for bacterial antigen, the other for acid phosphatase, it could be determined whether fluorescent macrophages and IAPh-cells were present in the same localization. The results of this comparison are listed in Table 1

Cells stained by the two methods occurred with the same localization in 69 kidneys with cortical cells (65.1 per cent of the total of 106 kidneys studied) in 45 kidneys with fornical cells (42.5 per cent) and in 11 kidneys with periabscess cells (10.4 per cent). The groupings of cells were often of a similar configuration in consecutive sections, indicating that the same cells had been stained by the two methods (Fig 1).

In a small number of kidneys fluorescent *macrophages* occurred, but *IAPh-cells were* not present in the same localization. Thus in two kidneys with cortical macrophages, corresponding IAPh-cells could not be demonstrated (1.9 per cent of the 106 kidneys studied). Four kidneys with fornical macrophages (3.8 per cent) and two with periabscess macrophages (1.9 per cent) did not reveal IAPh-cells in corresponding localizations.

On the other hand, the number of kidneys in which IAPh-cells occurred without fluorescent antigen-containing macrophages being demonstrable in the same localization was higher 15 kidneys with cortical IAPh-cells (14.2 per cent of 106 kidneys) 11 with fornical IAPh-cells (10.4 per cent) and 6 with IAPh-cells around abscesses (5.7 per cent) did not reveal fluorescent macrophages in these localizations.

DISCUSSION

In the digestion of phagocytized bacteria in the macrophage, cytoplasmic lysosomes fuse with phagocytic vacuoles, emptying their content of hydrolytic enzymes into the vacuoles. During this process, an increased enzymatic activity comprising acid phosphatase can be demonstrated in the cytoplasm of the macrophages (3) As the macrophage is a particularly rich source of lysosomes, this cell is apt to be visualized in staining for hydrolytic enzyme, especially acid phosphatase (13) By the method of *Burstone* the lysosomes were visualized as distinct red granules, which made the cells easily recognizable in the tissue. Comparison between the occurrence of bacterial antigen-containing macrophages and IAPh-cells had to be carried out using consecutive sections, as the two staining methods cannot be applied to the same section with any satisfactory result.

In this experimental model, interstitial cells with a positive granular reaction for acid phosphatase and macrophages containing bacterial antigen occurred in the same main localizations. In many cases, the same cells seemed to have been stained by the two methods. Only in a few cases, antigen-containing macrophages were revealed without IAPh cells being demonstrable in the same localizations, indicating that, by far most of the antigen-containing macrophages could in this study be visualized in sections stained for acid phosphatase. Thus, this observation in tissue sections is in agreement with previous *in vitro* experiments. On the other hand several more cases revealed IAPh-cells in localizations in which antigen-containing macrophages were not demonstrable. This was especially the case with cortical cells from the 2nd month onwards, and with periabscess cells during the first week. The reason for this might be that the reaction for acid phosphatase was positive for a longer period than that for bacterial antigen, or that some macrophages contained and digested material other than bacterial antigen, e.g necrotic debris.

If an analogy can be made to the more complicated conditions in human pyelonephritis, the method may prove to be of value in the selection of kidneys with a content of bacterial antigen in macrophages. Obviously a positive reaction for acid phosphatase would in itself by no means prove the presence of bacterial antigen in the macrophages, but a closer analysis of the content of these cells, e.g. with immunofluorescence using a panel of antibacterial antisera, would be indicated. Studies of the applicability of the method in the investigation of human chronic renal disease are in progress in our laboratory

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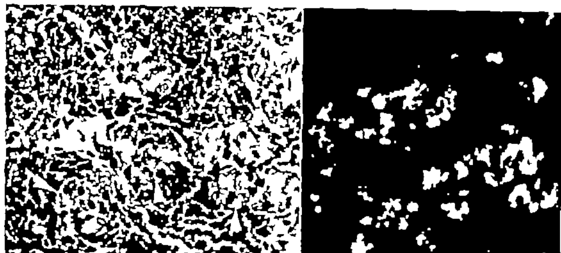


Fig 1 Left: groups of fornical cells (arrows) with fine cytoplasmic granules in section stained for acid phosphatase. The cytoplasm of these cells was light red owing to the numerous small, uniformly distributed granules, yielding a contrast to the violet-bluish of the surrounding tissue. Right: same groups of cells in a consecutive section stained with antiserum against *E. coli* 04. The fluorescent cytoplasm indicates that the cells are macrophages with phagocytized bacterial antigen. × 300.

similar to that of macrophages and contained acid phosphatase positive cytoplasmic granules of varying size from fine to coarse. Such cells were not found in non inflamed renal tissue, and owing to their characteristic appearance and localization they could not be mistaken for the normally occurring interstitial medullary cells which have a content of various enzymes, e.g. acid phosphatase (8)

Numerous interstitial acid phosphatase positive cells (hereafter referred to as IAPh-cells) occurred from the 2nd–3rd day with the same main localizations as the macrophages seen by immunofluorescence. In the *cortex* IAPh-cells were seen in 84 out of the 106 kidneys at first they were numerous, then occurring in decreasing numbers and gradually with fewer lysosomes. At the *fornix* groups of IAPh-cells were seen in 56 kidneys from the 3rd month with sparse cytoplasmic granulation. *Around abscesses* IAPh-cells were seen in 17 kidneys, generally in large numbers. Around abscesses, and often at the fornix, IAPh-cells generally contained smaller and more uniformly distributed lysosomes, than in the cortex.

Comparison between sections stained for bacterial antigen and for acid phosphatase

By comparing consecutive sections from the same kidney one of them stained for bacterial antigen, the other for acid phosphatase it could be determined whether fluorescent macrophages and IAPh-cells were present in the same localization. The results of this comparison are listed in Table 1

Cells stained by the two methods occurred with the same localization in 69 kidneys with cortical cells (65 1 per cent of the total of 106 kidneys studied) in 45 kidneys with fornical cells (42.5 per cent) and in 11 kidneys with periabscess cells (10 4 per cent) The groupings of cells were often of a similar configuration in consecutive sections indicating that the same cells had been stained by the two methods (Fig. 1)

In a small number of kidneys fluorescent macrophages occurred, but IAPh-cells were not present in the same localization. Thus, in two kidneys with cortical macrophages corresponding IAPh-cells could not be demonstrated (1.9 per cent of the 106 kidneys studied) Four kidneys with fornical macrophages (3 8 per cent) and two with periabscess macrophages (1.9 per cent) did not reveal IAPh-cells in corresponding localizations.

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MULTIPLE EPIPHYSEAL DYSPLASIA

2. *Morphological and Histochemical Investigation of Cartilage Matrix Particularly in the Pre-Calcification Stage*

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Rasmussen, P G Multiple epiphyseal dysplasia. 2. Morphological and histochemical investigation of cartilage matrix, particularly in the pre-calcification stage. Acta path. microbiol. scand. Sect A, 83 493–502, 1975.

The cartilage of epiphysis and cuboid bones from three Beagle puppies bred from parents with a constant heredity of multiple epiphyseal dysplasia, was examined macroscopically histomorphologically and histochemically The puppies were euthanized at the age of one week, three weeks and five weeks, respectively The investigation showed that foci of the disease develop in a broad subarticular zone where normally strong three-dimensional growth of the cartilage takes place. The first signs of the disease appeared about the time of birth and new foci reached climax when the puppies were about three weeks old. Histomorphological and histochemical examination of the cartilage showed that the disease developed in a sequence of confluent stages. In the initial stage there was an accumulation of abnormal cartilage matrix in the chondrocyte and/or chondrocyte lacunae. The abnormal substance consisted apparently of concentrated or free chondroitin sulphates not bound to protein. Then followed a second stage of liquefaction and coalescence of the abnormal cartilage matrix which formed cysts of different sizes, some of them visible macroscopically In the third stage of calcification of the cyst content and surrounding abnormal cartilage, it was found that the calcium was deposited in grains bound to chondroitin sulphates The results of the present study of the pre-calcification stage of the disease in Beagle dogs seem to be comparable with or equivalent to the results of the study of the post natal stage of the disease in children and may explain something of the prenatal pathogenesis in children.

Key words Epiphyseal dysplasia, multiple cartilage matrix; morphology; histochemistry

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In a recent radiological and histological study of material from diseased children and Beagle puppies (*Rasmussen & Reimann* 1973) it was found that the disease in children and puppies is apparently similar

Histological examination of material from diseased children has shown that the calcifications develop in spots of acellular substance which have been designated necrosis, oedema, cysts or mucoid degeneration of the cartilage (*Harris* 1935 *Burckhardt* 1938 *Bateman* 1939 *Hässler & Schallock* 1940, *Ford & Schneider* 1951 *Frank & Denny* 1954 *Yakovac* 1954 *Karlen & Cameron* 1957 *Gerring* 1965 *Kobylinski* 1967 *Cohen Solal et al* 1968) However all these histological investigations have been performed by

conventional histological methods on material which already showed pronounced abnormal calcification. From the histological study of six week-old Beagle puppies, it was evident that the calcification of the cartilage was secondary to abnormalities of the cartilage which had occurred at an early stage of development. Furthermore it was found radiologically that no abnormal calcification occurred in the Beagle puppies in the first three weeks of life.

Accordingly the aim of the present study was to examine the cartilage during the early pre-calcification stage of the disease. Furthermore the investigation was focused on the histochemistry of the heteroglycans of the cartilaginous matrix and the role of the heteroglycans in the different stages of the disease.

MATERIAL AND METHODS

The material consists of three Beagle puppies aged one week, three weeks and five weeks, respectively. The puppies were bred of parents with a constant heredity of multiple epiphyseal dysplasia. Specimens from the puppies were taken quickly after sodium mebumal anaesthesia and bleeding. The test specimens selected were the epiphyseal cartilage from humerus and femur as well as cuboid bone anlage from tarsus and the anlage of lumbar vertebrae.

Fixation. The specimens were cut into conveniently thin slices (2 mm) by razor blade. The fixatives were (1) Lillie's ethanol-formalin-acetic acid (*Lillie* 1965) (2) cetylpyridinium chloride-formaldehyde (*Williams & Jackso* 1956). Fixation was performed at 0–4° C for 24 hours. After embedding in paraffin, 5 micron sections were cut.

Staining and histochemical procedures. (1) Metachromatic staining with 0.1 per cent toluidine blue 0 (Merck) in 30 per cent ethanol (*Kramer & Windrum* 1955) staining time 1–2 minutes. (2) Metachromatic staining with 0.1 per cent toluidine blue in Walpole buffer at pH 1.0 and 2.0 and in McIlvaine buffer at pH 3.0, 4.0, 5.0 and 7.0. (3) Staining with 0.5 per cent alcian blue 8G (ICI) at pH 1.0 (adjusted by HCl) for 30 minutes. (4) Alcian blue 8G critical electrolyte concentration procedure (*Scott & Dorling* 1965, *Scott & Stockwell* 1967, *Scott et al.* 1968). (5) Digestion with testicular hyaluronidase (Fluka) (*Pearse* 1972). (6) Periodic acid-Schiff method for glycogen and other PAS-positive substances. Both De Tomasi's and Barger & de Lamater's Schiff reagents were used (*Pearse* 1968). Periodic acid was used as 1 per cent aqueous solution. Prior to the treatment with periodic acid, some sections were coated with celloidin in order to avoid loss of glycogen. (7) The von Kossa method for calcium deposits. (8) Decalcification of sections was carried out with equal parts of 2 per cent citric acid and 20 per cent sodium citrate pH 6.0, for 20 minutes (*Andersen & Balslev Jorgensen* 1960). Recalcification of decalcified sections was made with saturated $CaCl_2$ solution for 10–20 minutes.

RESULTS

Gross pathology. Examination of joints and cartilage from epiphysis and cuboid bones from the one-week-old puppy showed no macroscopical abnormalities. However in the cartilage of a few epiphyses from the three-week-old puppy cystic cavities 1 mm in diameter could be seen. The content was transparent and viscid. In many of the large joints of the five week-old puppy fluctuating opalic spots or cysts were found beneath the articular cartilage. The content of the cysts was white, liquid, and with fine grains of calcium deposits. In some joints, such large cysts could have burst and the content had become a thick white membrane inside the joint capsule. In such cases there was heavy inflammation of the joint.

Histomorphological and histochemical examination. The following description applies to the epiphyseal changes in a long tubular bone.

The epiphysis appeared to be segmented in four zones, as shown in Fig. 1. Zone 1 comprises the anlage of the articular cartilage proper. Zone 2 is the subarticular cartilage area situated below the articular cartilage zone and above the vascular cartilage canals. Only in this zone could the abnormal cartilaginous development be found although the abnormal development could be seen stretching to areas between the most superficially situated (as regards the joint cavity) vascular canals. Zone 3 is cartilage with normal appearance and containing the major vascular canals. Zone 4 is a normal growth plate (physis).

In the one-week-old puppy the abnormal

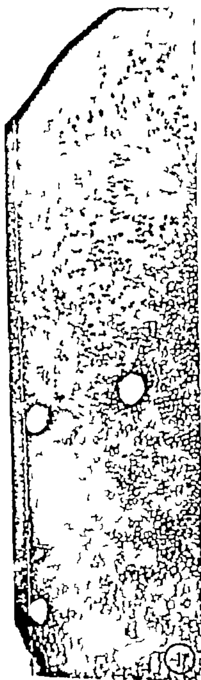
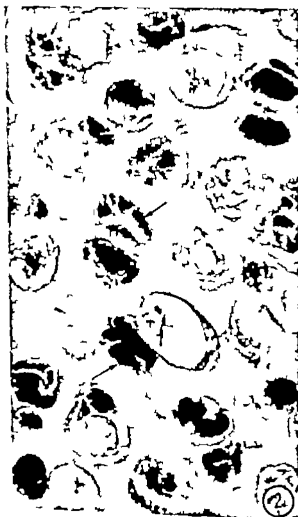


Fig 1 Section from the humeral condyle of a one-week old puppy showing the stratification in four zones Zone 1 – the anlage of the articular cartilage, Zone 2 – the zone in which the development of abnormal cartilage is observed Zone 3 – cartilage of normal appearance and containing the major vascular cartilage canals, Zone 4 – normal growth plate (physis) Note in zone 2 the accumulation of intralacunar flocculated material Toluidine blue in 30 per cent ethanol Magnification 32 ×

Fig 2 Epiphyseal zone 2 with accumulation of intralacunar floccular strong metachromatic material (arrows) which have forced the chondrocytes into a star-like appearance. Humeral condyle from one-week-old puppy Toluidine blue in 30 per cent ethanol. Magnification 400 ×



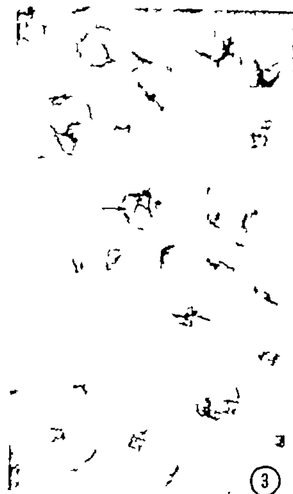


Fig 3 Same area as Fig. 2 with PAS-positive flocculated intralacunar material (arrow) and with glycogen in the cytoplasm of the star-shaped chondrocytes. Periodic acid-Schiff (de Tomasi's reagent) Magnification 400 ×

cartilage in zone 2 took the form of areas in which the chondrocytes were surrounded by accumulations of a substance situated between *the chondrocyte and the wall of the* cartilage cell lacuna. The accumulation had forced the chondrocytes into a star like appearance (Figs. 2 and 3) Due to this fact, it was difficult to observe whether some of the substance was also located intracellularly At that stage of development the chondrocytes of zone 2 were situated in small lacular groups separated by cartilage matrix of normal appearance. *The intralacunarly accumulated* substances appeared to be flocculated and showed strong metachromasia (Fig. 2) even at low pH values. The substance was stained with Alcian blue at pH 1 0 Using the Alcian blue *critical electrolyte* concentration method, the substance showed no alcianophilia at $MgCl_2$ concentrations >0.8 M Digestion with testicular hyaluronidase abolished the metachromasia and the alcianophilia. The same histochemical reactions were found in



Fig 4 Section from another area of humeral condyle of the one-week-old puppy showing the border line between the anlage of the articular cartilage (Jc) and the abnormal cartilage *of zone* 2. Note cyst formation (c) in lower right corner with "open up" *chondrocyte lacuna* from which flocculated material added to the content of the cyst (arrow) *Toluidine* blue in 30 per cent ethanol. Magnification 300 ×

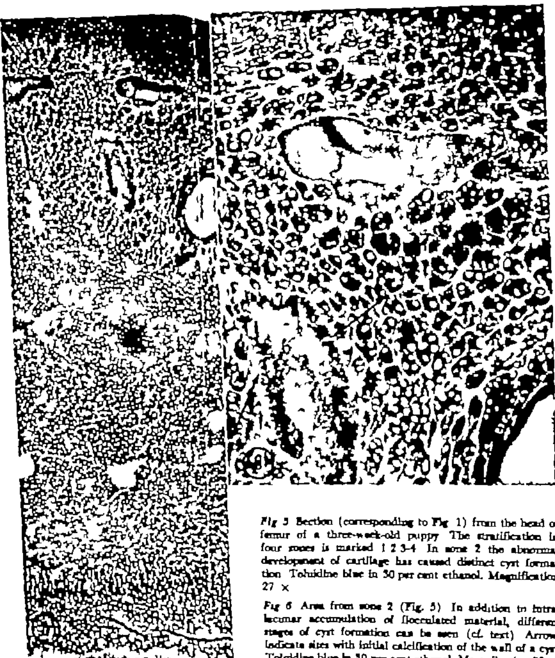


Fig. 5. Section (corresponding to Fig. 1) from the head of femur of a three-week-old puppy. The stratification in four zones is marked 1 2 3-4. In zone 2 the abnormal development of cartilage has caused distinct cyst formation. Toluidine blue in 30 per cent ethanol. Magnification 27 ×

Fig. 6. Area from zone 2 (Fig. 5). In addition to intralacunar accumulation of flocculated material, different stages of cyst formation can be seen (cf. text). Arrows indicate sites with initial calcification of the wall of a cyst. Toluidine blue in 30 per cent ethanol. Magnification 72 ×

the ordinary cartilage matrix of the four epiphyseal zones, although they did not attain the same degree of stainability. PAS-staining revealed maltase-resistant PAS-positivity in the intralacunar substance in accordance with the surrounding cartilage matrix (Fig. 3).

In some areas the cell nests seemed to have undergone regression with loss of cellular detail, and simultaneously the cartilage matrix between the chondrocyte lacunae was flocculated to an extent highly reminiscent of the flocculated intralacunar substance. In other

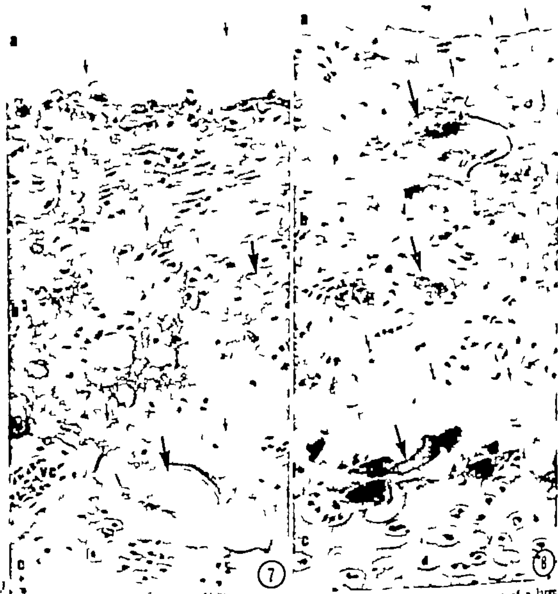


Fig 7 Section from the head of humerus from the five-week-old puppy showing a segment of a large cyst and its wall a = content of cyst, b = cyst wall with calculi of different sizes (small and large arrows) in the cartilage matrix, c = cartilage with normal appearance, vc = vascular canal. For further explanation, see text. Toluidine blue in 30 per cent ethanol. Magnification 192 ×

Fig 8 Similar segment of the same cystic structure after decalcification prior to staining with toluidine blue. Note the strong metachromasia corresponding to the positions of the calculi (small and large arrows) both in the content of the cyst and in th wall of the cyst. Decalcified section. Toluidine blue in 30 per cent ethanol Magnification 192 ×

areas the flocculated substance formed small cysts of acellular masses surrounded by intra lacunar cartilage matrix also showing signs of flocculation (Fig. 5) The cysts were confluent with similar material from "open up" cartilage cell lacunae (Fig 4 (arrows)) The content of the cysts showed the same histochemical reactions as mentioned above The best preservation of the floccular substance was obtained by using cetylpyridinium chlo-

ride as fixative. All chondrocytes of the four epiphyseal zones contained PAS-positive grains of glycogen.

There were considerably more of the changes mentioned in zone 2 (Fig 5) in the three-week-old puppy than in the one week old puppy Furthermore, cystic cavities of varying sizes (often visible macroscopically) were seen (Fig. 6) The flocculated content of the cysts showed decreased stainability but otherwise the same histochemical reactions as mentioned previously The cysts were mostly surrounded by abnormal cartilage characterized by large, often elongated, cell nests separated by abnormal wide areas of cartilage matrix. Intralacunar flocculated substance was always present in the abnormally large cell nests with star-formed cells in which signs of cellular disintegration could often be seen. Similar to what was found in the early cyst formation, the edges of the cysts showed the same "open up" of cartilage cell lacunae (Fig. 6)

At the edge of some of the large cysts there was a granular awlblade like configuration (Fig. 6 (arrows)) which showed weak ortochromatic (blue-green) staining with toluidine blue in ethanol, as well as strong PAS-positive reaction as compared to the ordinary cartilage matrix Decalcification of control sections revealed strong metachromasia in the formerly ortochromatic area but no decrease in PAS-positivity

Sections from the five-week-old puppy were characterized by pronounced granular calcification, both in the abnormal cartilage surrounding the cysts and in the content of the cysts. However notable cartilaginous changes in the chondrocyte lacunae, as well as early cyst formation as described in the one week-old and three-week-old puppies, could still be found in some epiphyses. Often the calcified cysts had attained a large size and had expanded extensively in zone 2 of the epiphysis. Due to the size the content of the cysts was sometimes lost during division of the specimens into thin slices prior to fixation.

In well-preserved sections, the cysts and their surroundings had a morphological appearance as shown in Fig 7 which was in accordance with the findings in a six week old and an eight week-old puppy (*Rasmussen & Reimann* 1973) The acellular mass of the cyst had granules of varying sizes and an intergranular substance without any definite structure. The granules showed distinct PAS-positivity were strongly stained by the von Kossa method, and showed no metachromasia with toluidine blue in ethanol. However decalcified sections (Fig 8) revealed strong metachromasia in the granules as compared to the intergranular substance, and showed all the histochemical reactions found in normal cartilage matrix. Recalcification of decalcified sections again showed disappearance of the strong metachromasia. The intergranular substance showed extremely weak stainability but otherwise the same histochemical reactions as normal cartilage. Surrounding the content of the cyst was a wide zone of abnormal calcified cartilage which showed the same histochemical reactions before and after decalcification as those mentioned above (Figs. 7 and 8) though the intergranular matrix showed higher stainability than the intergranular matrix in the cysts. The chondrocytes in the zone of abnormal calcified cartilage were of normal size Some of them showed signs of cellular disintegration, especially in the borderline to the cyst where the abnormal cartilage was more frayed and was often desquamated *into* the cyst cavity

Examination of affected cuboid bones in tarsus and spine from the one week-old, three-week-old and five-week-old puppies showed changes of the same kind as described in the epiphyses of the long tubular bones. The affected areas were situated in the zone below the articular cartilage, just as in the epiphysis.

DISCUSSION

Morphological examination of the pre and post-calcification stages of multiple epiphyseal dysplasia in Beagle dogs shows that the disease follows a definite pattern of develop-

ment. (a) Initially there is formation of an intralacunar and intracellular accumulation of floccular material which predominantly consists of chondroitin sulphate and glycoprotein. This is based on the following histochemical facts: the strong metachromasia even at low pH values, the alcianophilia at pH 1.0 and the digestibility with testicular hyaluronidase as well as the lack of alcianophilia at $MgCl_2$ concentration >0.8 M shows that the material belongs to the polycarboxy-sulphate type of glycosaminoglucoronoglycans (chondroitin-4 and/or chondroitin-6-sulphate). Furthermore, the maltase resistant PAS-positivity of the same material also indicates the presence of glycoprotein. (b) Then follows a liquefaction of the intralacunar flocculated material. This is based on the coalescence of the abnormal matrix to small cysts which enlarge continuously and merge into larger cysts of varying sizes, some visible macroscopically. The surrounding cartilage contains a characteristic arrangement of chondrocytes in large, often elongated, cell nests. This may be due to the liquefied matrix and to pressure from the liquid content of the cyst, probably aggravated by the movement of the puppy. (c) Finally calcification of the cyst content and surrounding cartilage develops.

Though gradually progressive, it seems reasonable to divide the disease into a sequence of development stages:

1. The initial stage, with floccular accumulation of chondroitin sulphate and glycoprotein within the chondrocyte lacunae.
2. The stage of liquefaction, with formation of cysts due to coalescence of the above-mentioned chondrocyte lacunae content and surrounding cartilage matrix.
3. The stage of calcification, with radiographhilacally stippled and mottled appearance.

It is evident from the present study that the target of multiple epiphyseal dysplasia in Beagle dogs is restricted to a definite subchondral zone of the epiphyses and cuboid bones. This is the zone where most of the normal epiphyseal growth takes place with high three-dimensional expansions (*McKibbin & Holdsworth* 1967). It should be noted that the target of achondroplasia is the epiphyseal growth plate (*Rubin* 1964), where the normal longitudinal growth of the tubular bone takes place, while the target of Morquio's disease is the articular cartilage (*Scheck & Haggerty* 1964) which has a special function. All these are hereditary diseases affecting a definite functional zone of the epiphysis and without primary effect on any other parts of the cartilage. Comparison of the above-mentioned target of these epiphyseal diseases serves to show that multiple epiphyseal dysplasia seems to be a separate genetic debris of the normal three-dimensional growth of the epiphysis and cuboid bones. In the initial stage, the flocculated and accumulated material inside the chondrocyte lacunae follows the histochemical reactions seen in normal young cartilage matrix. However, the material is flocculated as compared to normal intralacunar cartilage matrix with normal structure. This failure may be due to a predominant production of chondroitin sulphate not bound to a protein core, as is otherwise normal in cartilage matrix (*Mathews & Lozaityte* 1958, *Muir* 1958). The fact that the use of cetylpyridinium salt as fixative was superior to ethanol-formalin mixtures for preservation of the flocculated material is an indication of such a hypothesis, since cetylpyridinium salt forms highly insoluble complexes with the chondroitin sulphates. The strong alcianophilia of the flocculated material points in the same direction because Alcian blue seems to cause stronger staining of free chondroitin sulphates as compared to the staining of proteoglycans, due to a masking effect of their protein fraction (*Kobayashi* 1971). Several experiments on the genetic effect have shown that metabolism occurs in a stepwise fashion, with each stage controlled by a particular gen. Conversion of one compound into the next may be blocked by a mutation of the corresponding gene. This may lead to an

accumulation of the compound prior to the block and/or result in an inability to produce the compound including the normal metabolite distal to the block (*Horowitz* 1950). Multiple epiphyseal dysplasia seems to be such a mutagenic block of one step of normal synthesis of protein-chondroitin sulphates. On the other hand, as seen in Fig. 1, there seem to be few vascular canals in zone 2 of the epiphyses, and therefore nutritional factors must be taken into consideration. A deprivation of the nutrition may possibly explain both the changes in the initial stage and the disintegration of chondrocytes and the changes in the stage of cyst formation. Lysosomal enzyme activity may be the operative factor.

At the onset of the calcification stage, the disease takes on a new developmental pattern. The abnormal cartilage matrix is highly calcifiable, and this applies both to the content of the cysts and the wall of the cysts. As mentioned above, it is of interest that the present study seems to indicate that free chondroitin sulphates are produced intralacunarly during the initial stage. Furthermore, it would appear that during the calcification process the chondroitin sulphate has been accumulated in the calcified granules, while nothing or very little chondroitin sulphate is left in the intergranular substance to cause a metachromasia. The low stainability is noticed most clearly in the content of the cyst where the materials have also been diluted by a high degree of liquefaction. The present investigation may provide a reliable explanation for the development of the acellular substance designated necrosis, oedema, cysts or mucoid degeneration in children (authors mentioned in the introduction) and the two forms of pathological tissues demonstrated in the radiographical stippled and mottled calcifications in six-week-old and eight-week-old Beagle dogs (*Rasmussen & Reimann* 1973). The pathological tissue in children and dogs seems to be of the same nature and must be different steps in the stage of liquefaction and the stage of calcification reported in the present study.

The cartilage from the one-week-old puppy had only few foci following the initial stage changes, whereas cartilage from the three-week-old puppy had several foci of the initial stage and many foci of the liquefaction stage. The cartilage from the five-week-old puppy had also few foci showing the initial stage changes. It can be concluded from this that the disease in the different bones of the puppy begins at the time of birth, progressing to the fifth week of life, with a peak at the third week. Affected children are born with advanced calcification (*Rubin* 1964) and it seems probable that most of the foci with the initial stage changes found in children must have developed prenatally. In the puppies, only few foci with the initial stage changes were found as compared to many foci in the liquefaction stage. The initial stage seems to be short, whereas the liquefaction stage seems to last a relatively long time and continues during the calcification stage, since many cysts became enlarged in the three-week-old and particularly in the five-week-old puppies.

The osteogenesis in the puppy from birth to the age of three weeks seems related in every way to the osteogenesis in the prenatal child. The findings in the one-week-old and three-week-old puppies could well be equivalent to the changes in the prenatally diseased child.

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HISTOCHEMICAL DEMONSTRATION OF AN LNA SPLITTING ENZYME IN THE CEREBELLUM OF THE RAT

A Aminopeptidase Like Reaction Localized Selectively in the Granular Layer with Acid pH Optimum

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Albrechtsen, R. & Jensen, H. Histochemical demonstration of an LNA-splitting enzyme in the cerebellum of the rat. A aminopeptidase-like reaction localized selectively in the granular layer with acid pH optimum. Acta path. microbiol. scand. Sect. A 83 503–510 1975

Histochemical investigations of leucine aminopeptidase using LNA (L-leucyl-β-naphthylamide) as a substrate reveals a marked enzyme activity selectively localized to the granular layer with inconspicuous reaction in the stratum moleculare and the Purkinje cells in the rat cerebellum. The LNA-splitting enzyme differs from the well known leucine aminopeptidase (LAP) by its optimum at pH 5.5 The necessary long incubation period used in the present study and its local precipitation of the enzyme reaction product in the same place, like acid phosphatase, in the granular layer suggest a lysosomal localization. The functional role of the LNA-splitting enzyme has been discussed It is considered that it is involved not only in the protein transformation for synaptic function, but may perhaps also play an important pathogenic role in necrosis, atrophy or even autolysis.

Key words LNA-splitting enzyme cerebellum histochemistry rat

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Rat kidney is a very suitable organ for the demonstration of leucine aminopeptidase (LAP which has a high level of activity at pH 7.5 (23 25 36) The reaction product has a characteristic localization, as activity in the central parts of the renal cortex is far more marked than that in the more peripheral region this phenomenon can be used as a check of the reliability of the histochemical method (24) In the cerebral cortex, subventricular regions, periferal nerves and muscle end-plates similar reactions have been demonstrated biochemically at pH 7.5 (1 2) and histochemically at pH 6.8 (6 12) The presence of enzymes with proteolytic activity (8 19 37) is presumably of great importance for the function of the synapses (31) increase in the function of the nervous system leads to a demonstrable increase in proteolysis in the region of the synapses (12 13) A similar increase in proteolysis has been demonstrated histochemically in the muscle end plates during subneuronal stimulation, whereas there is no proteolysis when the muscle is resting (31)

TABLE 1 *Suitable Procedures for Preincubation*

Duration of preincubation 60 mins	Molarity	Amount per 100 ml	Buffer
$Mg_2SO_4 \cdot 7H_2O$	0.005 M	0.123 g	0.1 M acetate at pH 5.0, 5.5 6.5. 0.1 M phosphate at pH 7.5.
Cysteine (1) + EDTA (2)	1) 0.004 M 2) 0.05 M	1) 0.049 g 2) 1.86 g	
N-ethyl Maleimide	0.1 M	1.25 g	
Iodoacetate	0.1 M	2.08 g	

According to *Sylvén* (1968) the staining method used for the demonstration of LAP can be employed to give a selective demonstration of cathepsin B when carried out at pH 5.5 as the original aminopeptidase activity ceases at this pH (35 36)

In the present study we have investigated the leucine aminopeptidase activity in the cerebellum and kidneys of albino rats at various pH levels. Further classification of the enzyme activity was achieved by the addition of inhibitory or activating compounds.

MATERIAL AND METHODS

Pieces of fresh kidney and cerebellum from 15 albino rats, 5–6 month old, were removed after decapitation, and immediately frozen by submersion on the top of a metal tube surrounded by a freezing mixture of acetone/carbon dioxide snow (−80 C). The renal and cerebellar tissue was cut on a freezing microtome (Pearse SLEE) at −20° C, into 10 and 20 micron thick sections, respectively

The sections were studied for LAP activity by means of the method described by *Nachlas et al.* (1957) with L-leucyl-β-napthylamide HCl = LNA (no L-0376 Sigma) as specific substrate. In this reaction the substrate is hydrolysed with release of β-napthylamide, which is converted to a reddish azo-compound by coupling to Fast Blue B salt (no. 250 Michrome) The incubation times were 15 mins for kidney and 120 mins for cerebellum which gave the optimum of enzyme activity incubation temperature 37 C, and pH 5.0 5.5, 6.5 or 7.5. After incubation the sections were washed in distilled water and embedded in glycerine-gelatine. No fixation or counter staining was used.

The investigation was supplemented by of reactions which demonstrated [illegible] acceleration of the enzyme activity following pre-incubation in the following solutions (Table 1): EDTA + cysteine, N-ethylmaleimide (no. 3876 Sigma) magnesium sulphate, and iodoacetate. The preincubation time was 1 hour at 37 C. The sections were rinsed in buffer solution at the same pH as the incubation medium. Sections stained with haematoxylin-eosin were used for histological evaluation. Control sections were treated exactly as abov with the exception of the omission of L-leucyl-β-napthylamide. As check of the reproducibility of the enzyme reaction the LNA activity was investigated in a number of consecutive sections. The enzyme activity was evaluated semiquantitatively and the following grading was employed 0 no reaction. 1: slight reaction, the structure of the tissue was barely visible. 2 slight but definitely positive reaction 3 pronounces and distinct reaction without excessive staining of the tissue. 4 blurring of the tissue structure caused by excessive staining due to the high enzyme activity (*Jensen* 1972)

Fig 1 LNA splitting enzyme activity (black) localized to the deeper part of the renal cortex. Medulla (M) and glomeruli (G) show no enzyme activity (15 mins incubation, acetate buffer pH 7.5 without preincubation × 24)

Fig 1b LNA-splitting enzyme activity in the central area of the renal cortex, localized to the epithelium of the tubul (15 mins incubation, acetate buffer pH 7.5 without preincubation—same procedure as in fig 1 × 56)

Fig 1c LNA-splitting enzyme activity (black) in cerebellar cortex confined to stratum granulare Stratum moleculare (SM) and central nerve pathways show only weak enzyme acti ty (Preincubation 60 mins in N-ethyl-maleimide 120 mins incubation at pH 5.5 × 70)

1A M

1C SM

TABLE 2 *LNA splitting Enzyme Activity (at Various pH Levels and with Preactivati g and I hibiting Compounds) of the Granular Layer of Cerebellum and Tubuli of the Central Parts of the Renal Cortex*

Preincubation in	Granular layer of rat cerebellum pH				Tubuli of the central parts of renal cortex pH			
	5.0	5.5	6.5	7.5	5.0	5.5	6.5	7.5
$MgSO_4 \cdot 7H_2O$	1	2–3	1	0–1	0–1	1	2 3	2 3
Cysteine + EDTA	1	1–2	0–1	0–1	0–1	1	2	2
N-ethyl-Maleimide	1–2	3	1–2	0–1	0–1	1	2	2
Iodoacetate	1	2–3	0 1	0–1	0–1	1	2	2
Acetate or phosphate buffer	1–?	2–3	1	0–1	0–1	1–2	3	3

RESULTS

Kidney

There was marked enzyme activity in the kidney sections after 15 mins of incubation. The activity was mainly localized to the central parts of the renal cortex, while there was only weak activity in the peripheral parts (Fig 1 a) The collecting tubules of the medulla and the vessels showed no activity The reaction product, which consisted of a finegrained red precipitate, lay diffusely in the cytoplasm of the tubule cells, with a tendency to accumulation at the cell base (Fig 1 b)

Within the cytoplasm there were occasional vacuoles of about 1 micron diameter which showed marked enzyme activity The most marked activity was demonstrated in sections incubated at pH 6.5 and 7.5 *without* preincubation (Table 2) There was a considerable reduction in activity when the pH was altered to 5.5 and 5 0 The most marked fall in activity was observed in the sections which had been incubated in EDTA + cysteine N-ethylmaleimide and iodoacetate at pH 5.5 The enzyme activity was reduced throughout the sections, without any change in the relative intensity in the various areas of the tissue. The control sections, which had been treated in an identical manner but without the addition of specific substrate, were all negative.

Cerebellum

The activity was localized almost exclusively to the internal granular layer (Fig 1 c) and showed a sharp distinction from the Purkinje cell layer in which all activity was localized to the Bergmann s glial cells (Fig. 2 a) There was slight activity in the central white matter the choroid plexus and in the leptomeninges. The enzyme activity in the granular layer was confined to the course of the axons and could be seen as rows of small (approx ½ micron) concentric precipitates along the axon cylinders (Fig 2 b) Within the granular layer there were delimited areas of marked precipitation of reaction product, as evidence of intense enzyme activity These areas corresponded to the cerebellar glomeruli which have a very high synaptic density There was no activity in the nuclei of the granule cells.

Investigation of the enzyme activity at different pH revealed that the most marked reaction in the granular layer was found at pH 5.5 while there was less activity at pH 5 0, and activity had almost disappeared at pH 6.5 and 7.5 In contrast, there was an obvious increase in the activity in the Purkinje

Fig 2a. LNA-splitting enzyme activity in the granular layer confined to the course of the axons as rows of small concentric precipitates (same procedure as in fig 1c. × 160)

Fig 2b LNA-splitting enzyme activity after preincubation for 60 mins in $MgSO_4$. *Intense localization of the reaction product, presumably corresponding to the cerebellar glomeruli (G) No activity in the Purkinje cells (P) or the nuclei of the granule cells* (120 mins incubation at pH 5.5 × 210)

G

2A

G

P

cells and, even more marked the choroid plexus, at pH 6.5 and 7.5 There was slight inhibition of activity after preincubation in EDTA + cysteine most marked at pH 5.5 less at pH 6.5 and minimal at pH 7.5 The changes in enzyme activity were found to be distributed equally throughout the granular layer without any demonstrable difference in the loss of activity in the central and peripheral parts. There was no activity in the control sections.

All sections were evaluated and photographed immediately after the end of incubation. After 2–3 hours there was an obvious diffusion of the reaction product in the renal sections in the medulla, which otherwise showed no reaction. After 3 hours only slight diffusion was found in the molecular layer of cerebellum and no obvious fall in the colour of the reaction product could be demonstrated after 24 hours in the granular layer

DISCUSSION

We report here the demonstration of an LNA-splitting enzyme activity in the *cerebellum* localized to the granular layer and having greatest activity at pH 5.5 The first reports of investigations into LNA-splitting enzyme activity in the *cerebrum* to be found in the literature are the biochemical studies carried out by *Adams & Glenner* in 1962. These authors found the activity to be the same as that in the nerves, with a maximum at pH 7 0 and a reduction by 75–80 per cent at pH 5.5 They considered that *Nachlas et al.*'s (1957) histochemical method was inapplicable, as the diazonium salt (GARNET GB) was inhibited on incubation at pH 7 0 Other workers (*Benetato et al* 1965 *Gabrielescu et al.* 1966, 1971 *Poberai et al.* 1972) demonstrated by similar histochemical methods that there is an LNA splitting enzyme activity at pH 6.8 this is localized to the *cerebral* cortex, subventricular area, peripheral nerves and muscle end plates. In the last two areas the activity can be demonstrated only following stimulation (31)

Marks & Lajtha (1965) have reported a division of *cerebral* proteolytic enzymes (proteases) into acid and neutral groups in homogenates. These authors have shown that cathepsin D comprises 90 per cent of the acid proteases while the remaining 10 per cent consists of cathepsin B and C. They also state that there may in fact be other fractions, as the protein fractionation in their study was not complete.

The LNA-splitting enzyme activity is not only due to a pure leucine aminopeptidase (LAP) but there is presumably an additive effect of one or more proteolytic enzymes of the cathepsin group (10 12, 30 31 33 35, 36) In accordance with this view it has been shown by the film method (3) that there is a positive cathepsin reaction in the areas in which LNA-splitting enzyme activity has been demonstrated (12)

LNA splitting enzymes form a heterogenous group of amino-hydrolases (22, 30, 36) To these can be added other enzymes which according to some authors are capable of hydrolysing LNA (16, 31 33 35) including cathepsin B (33 35) which belongs to the amino-acyl napthylamidases, and cathepsin C (16, 31) while by contrast pure cathepsin B' (27) D and E cannot hydrolyse LNA.

In an opposition of the above mentioned, concerning which cathepsin is able to hydrolyse LNA, Pearse concludes (*Pearse* 1970 p. 970) that (citat) for the present the lysosomal LNAses, shown by use of LNA as substrate, do not represent any known lysosomal peptidase

A common feature of all cathepsins is their activation by cysteine + EDTA (6, 35 36) and, in the majority of cases, inhibition by iodoacetate and Mg^{++} or Mn^{++} (6, 30 36) This type of enzymes are localized to the lysosomes like for example, the acid phosphatases (32) and there will often be focal precipitation of the reaction product (11 26, 34) Further it is necessary to employ a somewhat longer incubation time than is the case for the aminopeptidases (36)

In the present study it was found necessary to use a relatively long incubation time to

demonstrate LNA-splitting activity which in the granular layer of cerebellum showed a distinct focal reaction product similar to the localization of acid phosphatases (4) In contrast to this, the enzyme reaction in the Purkinje cells and choroid plexus of the cerebellum were both diffuse arranged, and the reactions were presumably with the optimum at pH 6.8 due to LAP The difference between the activity of the LNA-splitting enzymes at different pH levels can perhaps be taken as evidence of the activity of a number of different enzyme systems (30)

The pH optimum of the LNA-splitting enzyme in the cerebellum presented here is not in accordance with that published hitherto (10 12 31) as these workers demonstrate in the cerebrum, muscle end-plates and nerves that the activity was maximum at pH 6.8, while we found a maximum activity at pH 5.5 in the granular layer

The weak enzyme activity observed in renal tissue at pH 5.5 when LAP activity would be excepted to have ceased, is according to *Sylven* (36) probably due to the effect of cathepsin B activity

On preincubation with EDTA + cysteine we found a weak inhibition of the LNA-splitting enzyme activity in the granular layer while *Benetato et al.* (1965) demonstrated that cysteine accelerated the activity of the cathepsins in the cerebrum.

It was impossible to demonstrate whether the LNA enzyme activity in the cerebellum was dependent on activation by Mg^{++} as this also failed with these ions in the kidney This could probably be explained by the Mg^{++} ions already present in both tissue specimens in optimal concentration by use of freeze sections (*Pearse* 1970 p 967)

The neuronal cathepsins and peptidases (15) are implicated not only in a functional role in protein transformation, as responsible for neuronal synaptic transmission (15 31) but also play an important role in various diseases of demyelinization (7 14) different forms of anoxia (20 26) and in the development of atrophy and even autolysis (11 32)

The granular layer is often selectively attacked under the above mentioned conditions, resulting in a severe fall in the number of granule cells (4 5 17 28, 29) Although the pathogenesis of the cell destruction of the granular layer is still unknown, it has been demonstrated in this study that the granular layer posses a high level of LNA-splitting enzyme activity which could explain why the cellular damage is localized selectively to this cell layer of the cerebellum. The LNA splitting enzyme activity differs from the well known leucine aminopeptidase (LAP) by its optimum at pH 5.5 and its focal precipitation of the reaction product, as acid phosphatases, suggest a lysosomal localization. Further study is therefore necessary concerning classification and specification of the demonstrated LNA-splitting enzyme, especially its relationship to cathepsins.

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THE POSSIBLE UTILIZATION OF NUCLEAR POCKETS ON LYMPHOCYTES IN THE DIAGNOSIS OF ENZOOTIC BOVINE LEUCOSIS

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Bloch, B., Bendixen, H. J. & Lund, E. The possible utilization of nuclear pockets on lymphocytes in the diagnosis of enzootic bovine leucosis. Acta path. microbiol. scand. Sect. A, 83 511–518, 1975.

A comparative study of the presence of lymphocytosis and nuclear pockets in lymphocytes was carried out using blood samples from animals belonging to herds with bovine enzootic leucosis and from herds free from leucosis. It was found that there was a correlation between the presence of such nuclear projections in a certain percentage of the lymphocytes and a state of lymphocytosis. As this correlation seemed to be independent of the aetiology of the lymphocytosis, it was not considered possible to employ the demonstration of nuclear pockets as a more specific tool in the diagnosis of bovine enzootic leucosis than the lymphocyte counts in themselves.

Key words: Leucosis, bovine, enzootic; lymphocytes; nuclear pockets.

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The diagnosis of bovine enzootic leucosis is in most countries based on histological and haematological methods. Employing these methods it is in most cases possible to arrive at a final diagnosis, at least when herd diagnosis is attempted, but differential diagnostic problems may sometimes be solved only by observing the herds over a period of up to three years. In the individual case it remains a serious drawback that the diagnosis is based on unspecific reactions, i.e. the lymphocyte counts. A more specific diagnostic method is therefore highly desirable.

Miller et al. (1969) and others have pointed out the possibility of utilizing the demonstration of nuclear vesicles (blebs, projections, pockets) in the lymphocytes of leucotic cattle, or cattle under suspicion for leucosis because of lymphocytosis, as a diagnostic means. The surveillance programme in Denmark regarding the bovine enzootic leucosis makes the Danish material especially well suited for a comparative study of blood cells using lymphocyte counts and evaluations of the occurrence of nuclear projections by means of electron microscopy.

In the following, the results obtained in such a study are reported.

MATERIAL AND METHODS

Blood samples. Blood samples which were taken from Danish cattle in connection with the routine surveillance programme were collected at random from three types of herds

1) from herds with bovine enzootic leucosis, 2) from herds under observation for leucosis because of the results of the haematological examinations previously carried out and 3) from herds declared free from enzootic leucosis.

One blood sample was taken for the conventional blood cell counts (*Bendixen* 1967) The blood was drawn as a 6–8 ml sample delivered into a tube containing 0.25 ml of a stabilizing and preserving fluid made up in the following way: 25 g of EDTA and 25 ml of a 36 per cent formalin solution made up to 250 ml using distilled water. The blood cells were counted 24–72 hours after the collection of the sample. The erythrocytes were haemolysed by addition of 0.2 ml of a per cent saponin solution, and the leucocytes were suspended by mixing 100 µl of the blood sample with 10 ml of a diluent which was made up in the following way 1800 g of NaCl, 120 ml of titriplex 3 140 ml of 36 per cent formalin and 300 ml of 0.5 mol trisbuffer made up to a 10 l stock solution. For use, the stock solution was diluted one to twenty. The leucocytes were counted electronically in a particle counter (Coulter) If the leucocyte counts were higher than 9000 cells per m^3 the cells were differentially counted in a smear preparation.

Another blood sample was taken for the electron microscopic examination. For this purpose the buffy coat was obtained by following a procedure according to *Weber et al.* (1975) with modification.

A sample of 5 ml blood was drawn into a tube which contained 7.5 mg EDTA in 0.5 ml PBS (phosphate buffered saline) The sample was centrifuged at around 3000 r.p.m. (1500 g) for 15 min immediately after it had been drawn. The plasma was pipetted off and about one ml of 2.5 per cent solution of glutaraldehyde in 0.1 M phosphate buffer of pH 7.3 was carefully poured along the inner side of the tube without disturbing the buffy coat. After 30–60 min, the fixative was removed and the buffy coat was transferred to a tube containing 3 ml of the glutaraldehyde solution. After a further 3-hour-period, the fixative was removed and the buffy coat rinsed several times during the next three hours in 0.1 M phosphate buffer containing 10 per cent of saccharose. The fixed samples were kept in the final rinsing volume until postfixation and embedding.

Electron Microscopy

After transport and sometimes storage in the rinsing solution, the buffy coats were cut in smaller pieces with—for purposes of orientation—a triangular facet parallel to the sedimentation plane and rectangular sides containing the different layers of the buffy coat. The pieces were postfixed overnight at 4 C in phosphate buffer (pH 7.3) containing 1 per cent of osmium tetroxide (OsO_4) After dehydration in acetone, the pieces were transferred through styrene to Vestopal-W (Serva) in which they were embedded.

After polymerization, the blocks were sectioned parallel to the rectangular facets. The sections containing the leucocyte layer situated between the erythrocyte layer and the thrombocyte layer were stained in uranyl acetate and lead citrate. The sections were examined in JEM 100 B microscope and photographed t 2500 times magnification The number of nuclear pockets (see below) and the number of lymphocytes were counted on micrographs representing the whole lymphocyte layer (Fig. 1) In each preparation, a minimum of 250 cells was examined.

Description of the Nuclear Pockets

The term nuclear pocket (nuclear vesicle, bleb, hollow appendage) is in this report employed to describe a pocket-shaped projection on the surface of the nucleus. The nuclear material forming the prominating wall of the pocket is on both sides lined with the membranes of the nuclear envelope in accordance with *Bessis* (1973) The thickness of the sheet of nuclear material is around 35 nm, measured between the lining inner membranes.

The pockets are filled with cytoplasmic material which apparently often is somewhat autolysed. In the present work, only projections which are observed as arcs with both ends attached to the nucleus and completely surrounding cytoplasmic material are registered as nuclear pockets. Lymphocytes with such nuclear pockets are shown in Fig. 2 and 3

RESULTS

As a result of the routine surveillance programme, the herds may be divided into three categories.

The *first* consists of herds which were regarded as affected by enzootic leucosis or were under observation due to the discovery of persistent lymphocytosis in some animals. The second category consists of herds under observation for enzootic leucosis, either because one leucotic tumour case was found or because lymphocytosis was discovered in some animals. The third category consists of herds

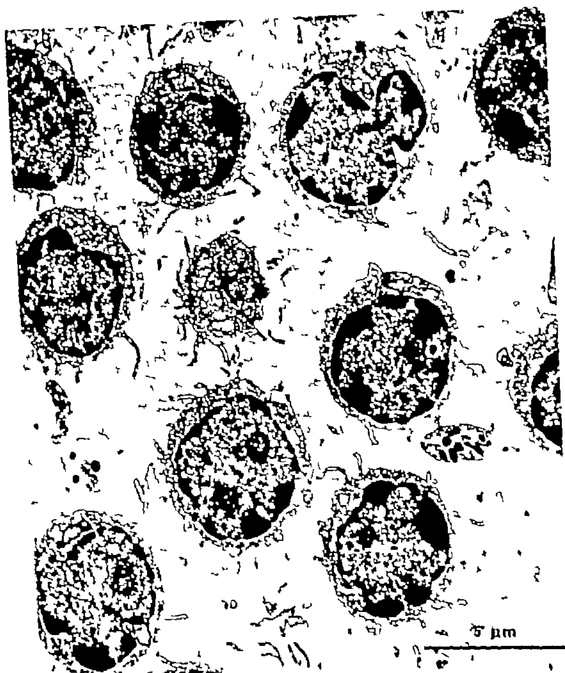


Fig 1 Example of micrographs of the lymphocyte layer from which the number of lymphocytes containing nuclear pockets were counted

considered free from leucosis. This last group comprises some herds which have been under observation because of lymphocytosis, but which were later regarded as free from leucosis because of the transient nature of the lymphocytosis.

In the present material, blood samples were collected from 25 herds. Six out of these were



Fig. 2 Lymphocyte containing nuclear pockets

leucotic herds: in 2 herds single tumour cases had occurred and the herds were under observation for enzootic leucosis, 6 herds were under observation and later defined as leucotic herds, one herd remained under observation, 4 herds included animals with transient lymphocytosis of a degree sufficient to require short term observation. The rest of the 25 herds had always been considered free from leucosis and remained so.



Fig. 3 Lymphocyte containing nuclear pockets.

Fig. 4 shows a plot of the results obtained when blood samples were examined using lymphocyte counts and electronmicroscopy. The results were divided into three groups. The first group consisted of blood samples from the herds which were leucotic or were under observation due to high lymphocyte counts at the time when the examined blood samples were drawn and later were recognized as leucotic herds. The second group



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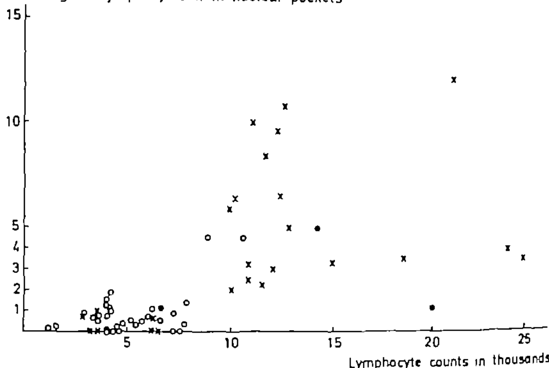


Fig 4 The number of lymphocytes in thousands per mm³ is plotted against the percentage of lymphocytes containing nuclear projections in blood samples taken simultaneously from the same animals. The crosses indicate blood samples from leukotic herds. The circles indicate blood samples from normal herds without enzootic leukosis, and the filled circles blood samples from herds under observation for leukosis

consisted of samples from herds under observation for enzootic leucosis, and the third group was from herds accepted to be without leucosis. This last group included such herds as were under observation at the time when the samples used in the present report were collected, but later were accepted as free from leucosis.

As may be seen from Fig 4 there is a considerable spread of the results, but the essential point is that lymphocytosis and presence of nuclear pockets in a certain percentage of the cells seem to be correlated, irrespective of the group to which the blood samples belong

The single cases of high lymphocyte counts in normal, not leucotic herds are of special interest, and information on these is collected in Table 1 As may be seen from this table, 9 out of 11 animals in which lymphocytosis recently had been manifest and in which lymphocytosis occasionally still persisted, had also nuclear pockets in a number of their lymphocytes

DISCUSSION

Nuclear projections in human leukaemic cells were first described by *Epstein & Achong* (1965) but such formations have also been found in cells from normal individuals as well as in cells from persons suffering from a variety of diseases (*Ahearn et al.* 1967 *Mehas* 1966 and *Smith & O'Hara* 1967) In spite of these observations, the hypothesis was put forward that corresponding ultrastructures in bovine lymphocytes might be of specific origin and related to the activity of a leukaemogenic agent. This hypothesis was suggested by *Miller et al* (1969) and later by *Weber et al.* (1973)

It appears from the results of the present

TABLE 1. *Results of the Examination of the Total Number of Blood Samples Obtained from the Individual Cows with Transient Lymphocytosis in Herds without Enzootic Leucosis*

Cow No.	*Date for blood sampling*	Total lymphocyte count per mm³	Percentage of lymphocytes with nuclear pockets	Description
1	Febr. 3	13000		Transient lymphocytose
	April 13	7500	0.6	Herd under observation until May 5 and then registered as leucosis-free
2	Jan. 20	10700		Transient lymphocytosis.
	March 9	10600		Herd under observation until
	March 21	8400	1.5	October 1972.
3	Jan. 11	11800		Single animal with lymphocytosis.
	Febr. 21	9900		*Observation discontinued November*
	March 23	8700	4.5	and herd registered as leucosis-free.
4	Febr. 21	7800		Single animal with lymphocytosis
	March 23	7300	0.5	during a short period in leucosis-free herd.
5	Jan. 13	7500		Single animal with transient
	March 17	11000		lymphocytosis in leucosis-free herd.
	March 22	7500	0.0	
6	Jan. 2	6800		*A few animals with transient*
	March 23	4600	0.0	lymphocytosis in leucosis-free herd.
	Jan. 2	7100		
7	March 23	5200	0.5	
8	Febr. 9	12100		Leucosis-free herd with 3 animals
	March 23	5600	0.4	with neutrophilia and lymphocytosis.
9	Febr. 9	5500		On April 25 all three animals had
	March 23	4100	1.1	normal blood values.
10	*Febr. 9*	6000		
	March 23	4400	1.8 (also in monocytes)	
11	Nov. 30	11800		A cow from a leucosis-free herd.
	Febr. 2	12600		A foreign body was removed by
	March 16	12700		operation and in this connection a
	April 13	10600	4.5	pyemia" developed. No signs of leucosis.

work (Fig. 4) that there is a correlation between the presence of nuclear pockets and the state of lymphocytosis whenever pockets are present in more than 2 per cent of the lymphocytes. This correlation may be found whether herds are affected by enzootic leucosis, or are *suspected to be affected*, or are free from this disease. Consequently it does not seem possible to use the demonstration of nuclear pockets in an attempt to improve the specificity of the diagnosis in the individual case of lymphocytosis. Even if only a quantitative difference rather than the qualitative difference existed, which was originally hoped, the search for nuclear pockets might still have been useful in the diagnosis. *The individual* cases of lymphocytosis found in the normal herds where nuclear pockets were present in a certain percentage of the lymphocytes exclude, however such a possibility

The samples from normal leucosis-free herds in which transient lymphocytosis was observed, were selected at random in the total Danish material. Since nine out of the first examined 11 cases had the same correlation between lymphocytosis and the presence of nuclear pockets as the material from the leucotic herds, and since 2 out of these 9 cases had more than 2 per cent of lymphocytes containing nuclear pockets, further investigation was considered unnecessary. In addition the normal herds included a number of cases in which increased numbers of lymphocytes were associated with a presence of blebs in a low percentage of the lymphocytes. It is also of interest to note, that nuclear projections may be found in leucocytes other than lymphocytes. Thus, projections were found in the monocytes of three animals out of the total of 63 animals examined electronmicroscopically in the present study. One animal was from an "observation" herd, and two animals were from leucosis-free herds.

Thus, the general conclusion seems to be that nuclear projections are formed under different circumstances, when the production of lymphocytes is stimulated and the number of circulating lymphocytes is increased. It appears that projections are often formed in lymphocytes during this stage of accelerated formation and that the presence of projections should be taken as a sign of this activity rather than as an indication of a specific stimulation by a leukaemogenic agent.

The observations by several authors that nuclear projections are closely related to leucosis are still correct in so far as bovine leucosis is of lymphocytic nature and involves a long sub-clinical phase of persistent lymphocytosis. In herds and cattle populations where this disease is widely distributed, there may be only a very small percentage of animals with nuclear projections in the lymphocytes which not suffer from leucosis. However in areas where enzootic leucosis is rare the situation is quite different. Thus the counting of nuclear projections seems only to be a more complicated way of demonstrating a condition already revealed by the lymphocyte counts.

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DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY IN HEALTHY AND DISEASED HUMAN LIVER TISSUE

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Hågerstrand, I. Distribution of alkaline phosphatase activity in healthy and diseased human liver tissue. Acta path. microbiol. scand. Sect. A, 85: 519–526, 1875.

Histochemical demonstration of the alkaline phosphatase activity with an azo-dye method in human livers showed that the activity, which is normally localized to endothelial cells of portal and central veins as well as of sinusoids around the central veins and to a lesser extent of sinusoids around the portal connective tissue, is increased in various liver diseases. Appreciable canalicular activity is rare, and when it occurs it is most prominent in parenchyma involved by malignant tumours.

Key words: Liver; enzymology; phosphatases.

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The alkaline phosphatase activity in normal human liver according to the Gomori's method as well as azo-coupling methods is known to occur in the sinusoids, in the endothelium of central and periportal veins, in the walls of hepatic arteries and sometimes locally in bile canaliculi. No activity is found in the bile duct epithelium or in the portal connective tissue (4, 5, 8, 11, 12). Some fairly recent reports are available on enzyme histochemical patterns of human liver in obstructive jaundice (2, 8). Gomori's method as well as azo-dye techniques for alkaline phosphatase have demonstrated more intense and extensive staining in the same structures as in normal human liver tissue. In 1947 *Sherlock & Walsh* (11) described the distribution of alkaline phosphatase in normal and diseased human livers (Gomori's method). They found the sinusoidal activity to be increased in various diseases, and in obstructive jaundice and in generalized bone disease (tumour metastases in bone, Paget's disease of bone) also canalicular activity was increased. *Cleveland et al.* in 1950 (4) with the same method likewise described increase in alkaline phosphatase activity in the sinusoids in hepatitis, in cirrhosis and in obstructive jaundice. Focal activity of canaliculi was described in normal specimens, and increased extent of canalicular activity was observed in hepatitis and cirrhosis, but still more in biliary obstruction.

For some years we have used enzyme histochemical methods in the study of human liver biopsy specimens in an endeavour to assess their diagnostic value as well as to obtain human material comparable with that of different animal species used in experimental enzyme histochemical studies (1, 6). This paper compares the distribution of

TABLE 1 *Survey of the Biopsy Material*

Diagnoses and number of specimens		Subdiagnoses and number of specimens		Remarks
Acute hepatitis	161	With the picture of acute viral hepatitis	137	Mainly Au-positive hepatitis including 11 cases with relapsing hepatitis 3 cases of mononucleosis
		Alcoholic hepatitis	8	+ *16* specimens among the cirrhoses with alcoholic hepatitis
		Known drug-induced hepatitis	6	Sulfa, chlorpromazine, erythromycin, acetphenolisatin, Bilharzia?
		Un-classified	10	
Chronic hepatitis	95	Chronic persistent or non-specific reactive	39	
		Chronic aggressive	35	+ 4 specimens among patients with cirrhosis
		Primary biliary cirrhosis	2	
		Granulomatous	9	
		Echinococcus	1	
		Non-classified	9	
Extra-hepatic obstruction	68			
Cirrhosis	126			
Steatosis	109			
Malignant diseases	50			
Alpha-1-antitrypsin deposits	3			+ 6 (2 of those with malignant diseases, 2 with cirrhosis, 1 with amyloidosis in the group of various changes, 1 with steatosis)
Various changes	72			
Normal histology + canalicular alkaline phosphatase activity	34			
Normal specimens	102			

alkaline phosphatase (E.C.3.1.3.1 sodium *naphthol-AS-TR*-alkaline phosphatase demonstrated by the azo-dye method) in diseased and normal human liver tissue.

MATERIAL AND METHODS

The material consisted of 820 human liver biopsy specimens obtained in 1967 to 1973. Most biopsies were performed by the Menghini technique. The specimens were kept at 0° C for 10 to 20 minutes and then frozen with carbon dioxide snow or with propane cooled with liquid nitrogen. They were then stored at 70° C until cryostat sections (about 6 microns thick) were cut and stained for enzyme activities. The remaining part of the frozen specimen was later thawed, fixed in formalin and embedded in paraffin. Paraffin sections were then stained with haematoxylin-erythrosin, van Gieson for collagen fibers, with silver for reticulin fibers, with periodic-acid-Schiff (PAS) according to Mac Manus (with and without diastase-digestion) and Prussian Blue for iron. Also unfixed, cryostat sections were stained with PAS. Alkaline phosphatase method. Cryostat sections were fixed for 10 minutes at +1° C in a solution consisting of 10 ml conc formalin, 20 ml 0.1 M phosphate buffer pH 7.4 70 ml distilled water and then rinsed for 3 minutes at +1° C in 0.1 M phosphate buffer pH 7.4. The sections were afterwards incubated for 30 minutes at room temperature in 3 mg naphthol-AS-TR phosphate (sodium salt) dissolved in 25 ml distilled water and mixed with 30 mg Fast Red

TABLE 2. *Survey of the Results*

Diagnosis	Alkaline phosphatase activity in		
	sinusoids	canaliculi	bile duct epithelium
Normal specimens	+	0 or (+)	0
Acute hepatitis	+ + +	0 or (+)	0
Chronic persistent or non-specific reactive hepatitis	+	0 or (+)	0
Chronic aggressive hepatitis	+ + +	0 to +	0
Extra-hepatic obstruction	+ + +	0 to +	0
Cirrhosis	+ to + + +	0 to +	0
Steatosis	+	0 or (+)	0
Malignant diseases	+ to + + +	0 to + + +	0
Alpha-1-antitrypsin deposits	+	0 to + + +	0

0 = no activity
(+) to + + + = increasing amount of staining product.

TR dissolved in 25 ml 0.1 M tris-(hydroxy-methyl-amino-methan) buffer pH 9.0 After incubation the sections were rinsed for a short time in distilled water and the nuclei were stained for 45 seconds in haemalum. It is essential to dissolve the diazonium salt in the buffer solution before preparing the final solution, for otherwise the diazonium salt is apt to be decomposed with staining of the solution as a result. The incubation solution is initially waterclear but becomes yellow during incubation. —Sigma chemical company St. Louis, Missouri, U.S.A., supplied the chemicals. One section from a normal rat liver served as a control at each incubation for alkaline phosphatase Incubations without substrate, sections subjected to enzyme inactivation or enzyme inhibitors were not regularly used

The distribution of the diagnoses among the specimens is given in Table 1

RESULTS

See Table 2.

Normal specimens. The red azo-dye indicating alkaline phosphatase activity was found in the endothelial lining of central and portal veins and hepatic arteries as well as along sinusoids around central veins and portal connective tissue. The sinusoidal staining was most extensive around the central veins. In about 20 of the 102 specimens few barely discernible red-stained canaliculi were detected. No activity was found in bile duct epithelium. Fig. 1

Acute hepatitis Most of these specimens showed a more extensive staining along sinusoids than normal tissue. The increase was correlated with the severity of the morphologic changes. Staining was thus heavier in acute cholestatic viral hepatitis than in mild hepatitis and viral hepatitis in the resolution stage. In some of the 161 cases a few positive canaliculi appeared as in the normal specimens, but in 5 specimens there was a more extensive though thin canalicular network. Fig. 2

Chronic hepatitis In the cases diagnosed as *chronic persistent* or *non specific reactive hepatitis* the specimens showed alkaline phosphatase activity corresponding to that of normal liver tissue the same applied for *granulomatous hepatitis* and *non-classified chronic hepatitis*. However in the periphery of granulomas an increased azo-dye product was sometimes seen both along sinusoids and canaliculi (Fig 3) Barely discernible stained canaliculi were found focally in 9 of the 39 specimens and a diffuse positive, slender net work of canaliculi in further 6 The specimens interpreted as *chronic aggressive hepatitis* or *primary biliary cirrhosis* showed increased intensity and extent of azo-dye product along the vascular channels. The intensity of staining varied between different hepatic lobules as well as within individual lobules. A strong dye deposit was often seen in the

vicinity of proliferating collagen fibres in parenchyma becoming cirrhotic. About 10 of the 37 specimens showed slender positive canaliculi, but no complete canalicular net work. Only in some specimens did the canali cular activity appear abnormally high. Fig 4

Some of the 68 specimens from patients with verified *extra-hepatic obstruction* have been used in an earlier investigation (2) The alkaline phosphatase activity was found to be increased along the walls of vessels, and especially of central veins and surrounding sinusoids. The larger the number of bile casts and the amount of pigment, the more intense was the staining In 29 subjects the obstruc tion was due to malignant growths in 39 to stones. Three specimens from the former group and 5 from the latter contained a continuous, rather intensely stained product depicting canaliculi. In addition, 19 speci- mens (7 from the malignant group) showed focal insignificantly increased canalicular ac tivity Fig 5

A diagnosis of *cirrhosis* was made in 126 specimens. Various patterns of alkaline phos- phatase activity were obtained. Sometimes staining was only weak and confined to the vessel walls, sometimes there was an irregular increase in dye deposits with or without pat chy canalicular staining This description might hold for different specimens as well as for the differences within and between the pseudolobules of a given specimen. 38 speci mens contained patchy canalicular deposits, but only 5 exhibited a continuous canalicular net work. A varying amount of dye deposits appeared in the connective tissue in 25 cases, all of which showed histological signs of active formation of fibrous tissue (Fig 6)

Steatosis Specimens with steatosis alone or combined with mild cholestasis were included. Many of the patients were known to be heavy drinkers. A moderate increase in stain- ing product along vessel walls was observed in some specimens with or without co-existing cholestasis. Insignificant, scattered and deli- cate canaliculi were seen in 15 specimens and a somewhat more evident canalicular system in 6 further cases.

Malignant diseases 50 specimens showed primary or secondary malignant growths of varying, though mainly epithelial origin. Some of these specimens have been used in an earlier investigation (1) The sinusoidal deposits of the enzyme reaction appeared in- creased in 36 specimens, but were usually confined to sinusoids adjacent to malignant tissue. In 38 specimens azo-dye depicted both perifocal and remote canalicular structures. The canaliculi, which were intensely stained, appeared continuous and thickened. No cor relation was found between the size or the histologic type of the tumour and the canali- cular alkaline phosphatase activity Fig. 7

Alpha 1-antitrypsin deposits Of 9 speci- mens showing Mac Manus-positive, diastase resistant globules of the hepatocytes (10) red azo-dye was clearly visible along canaliculi in 7 2 of these specimens are included in the group of malignant diseases and ? in that of cirrhosis. One specimen (in steatosis group) showed rather advanced fatty changes, one in the group of various changes amyloidosis (Fig 8) —One subject (a boy with neonatal jaundice) was biopsied twice at an interval of 5 years. Mac Manus-positive globules were found on both occasions, but no canalicular activity of alkaline phospha-

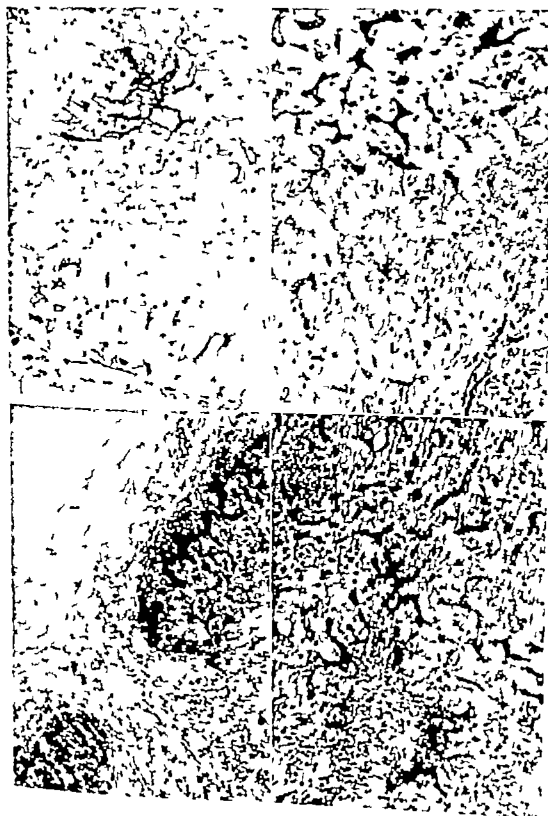
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Fig 1 T6871/71 Healthy human liver with nor mal alkaline phosphatase activity in endothelial cells of central eins and adjacent sinusoids (upper part) as well as small essels of portal area and adjacent sinusoids (lowe part) × 153.

Fig 2 T5456/75 Ac t ral hepatitis with in- creased staining reaction of alkaline phosphatase in sinusoids, mainly in central parts of the hepatic lobule (upper part portal zone at bottom) × 153.

Fig 3 T?359/69 Wall of echinococcus cyst upper left corner with an usually intense staining of alkaline phosphatase activity in sinusoids as well as canalicul of isolated parenchymal nodules, while the rest of the liver parenchyma showed pattern like that n Fig 1 153

Fig 4 T18822/71 Chronic aggressive hepatitis with irregularly increased sinusoidal activity of alkaline phosphatase Portal area in lower part. × 153.


1
2



5
8

sue. Three specimens in the material were from serologically known heterozygous individuals, but no classical globules were found in their hepatocytes. In 2 of those 3 specimens, however staining of alkaline phosphatase activity showed a canalicular net work. One specimen is included under the heading of "various changes" the other 2 in the group of "normal except for canalicular alkaline phosphatase activity".

Miscellaneous histologic findings (insignificant cholestasis, other pigment deposits, slight fibrosis) were grouped under the heading of *various changes*. Most specimens showed only negligible morphological changes. Usually the alkaline phosphatase pattern was normal, yet as many as 15 specimens with clear canalicular activity were assigned to this group.

Finally there was a group of 34 specimens of normal histologic appearance, but with an increased canalicular net-work when stained for alkaline phosphatase. Two of these specimens have recently been discussed (3). They derived from subjects with renal carcinoma without liver metastases. The whole group will be analysed in a further study.

Fig. 5. T3104/68. Increase of sinusoidal alkaline phosphatase activity in a subject with choledocholithiasis. Part of portal zone in lower part. × 153

Fig. 6. T7143/69. From a specimen with cirrhosis. Fibrosis with vessels and alkaline phosphatase activity in the connective tissue in lower part. Sinusoidal activity of varying intensity. × 153

Fig. 7. T4887/68. Liver parenchyma with canalicular alkaline phosphatase activity from a patient with adenoacanthoma of the gallbladder and metastatic growth in the current biopsy specimen. Portal zone to the left. No tumour growth is seen in the picture. × 153

Fig. 8. T10334/69. Liver parenchyma with amyloid deposits (= pale areas) and canalicular activity of alkaline phosphatase. Besides globules as described in alpha-1-antitrypsin deficiency were found. The patient (still alive 1975) has healed pulmonary tuberculosis and has been operated and X-rayed because of mammary carcinoma. × 153

DISCUSSION

The azo-dye technique used for histochemical demonstration of alkaline phosphatase activity proved both simple and reproducible without disturbing or irregular artefacts. As known the Gomori methods have certain disadvantages (9). We have tried several methods for demonstrating alkaline phosphatase (Gomori methods according to the calcium cobolt as well as lead variation with beta glycerophosphate as substrate azo-dye method with alfa-naphthyl phosphate and naphthol-AS-TR phosphate as substrates) simultaneously in some liver specimens. All gave largely the same results.

Alkaline phosphatase activity of healthy human liver tissue is confined to vessel walls and sinusoids, in contrast with the mainly canalicular localization in many other species (dog, pig). The activity along sinusoids is localized to the cells lining the sinusoids, which *inter alia* has been proved by staining of fine needle aspirations. A layer of hepatocytes which is only one cell thick derives from one cell plate (7) and shows no activity when the aspiration is obtained from a normal liver.

The parenchyma of a diseased liver reacts mainly with an increase in alkaline phosphatase staining in endothelial cells. Such an increased staining is a good indicator of the activity of any liver disease. A patchy canalicular staining was fairly common in chronic aggressive hepatitis, in cirrhosis, in extrahepatic biliary obstruction, but more pronounced canalicular staining appeared mainly in the specimens containing malignant tissue. An isolated, diffuse canalicular reaction and otherwise normal liver histology was a rare finding which might be of diagnostic significance. Two of the specimens with this pattern were obtained from patients with renal carcinoma without liver metastases, which indicates a remote tumour effect on the liver (3).

The findings of *Sherlock & Walshe* (11) and of *Cleveland et al.* (4) performed with the Gomori calcium-cobolt method agree

well with our findings with azo-method. The differences in the descriptions can be referred mainly to artefacts of the Gomori method, such as staining of nuclei and bile pigments. It is interesting to note that at least 2 of *Sherlock & Walshe's* patients with canalicular activity had malignant tumours.

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ON HISTOCHEMICAL ENZYME CHANGES IN ASSOCIATION WITH CANALICULAR ACTIVITY OF ALKALINE PHOSPHATASE IN HUMAN LIVER

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Hägerstrand, I On histochemical enzyme changes in association with canalicular activity of alkaline phosphatase in human liver Acta path. microbiol. scand. Sect. A, 83 527–533 1975.

56 human liver biopsy specimens with insignificant or no histological changes, but with abnormally strong canalicular alkaline phosphatase activity were studied histochemically for other enzyme changes. In comparison with normal specimens, more extensive and increased canalicular activity of gamma-glutamyl transferase, and increase of canalicular leucine aminopeptidase, was found, while the sinusoidal activity of the latter enzyme was decreased. Staining for adenosine triphosphatase regularly disclosed the normal pattern of sinusoidal and canalicular activity The lysosomal enzymes, acid phosphatase and beta-glucuronidase, stained more intensely than ordinarily while the reactions for enzymes present in the cytosol (lactic dehydrogenase) in the mitochondria (succinic dehydrogenase, monoamine oxidase) and in the endoplasmic reticulum (glucose-6-phosphatase) were normal.

Key words: Liver enzymology

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The distribution of alkaline phosphatase activity in healthy and diseased human livers has been described previously (9 10) The sinusoidal activity appeared to be abnormally high in various liver diseases, while the canalicular activity was appreciable mainly in malignant tumours with or without growth in the liver in collagen diseases (rheumatoid arthritis, temporal arteritis) and in specimens with alpha-1-antitrypsin deposits. Often liver specimens with canalicular alkaline phosphatase activity showed no changes in sections stained with conventional methods.

Alkaline phosphatase is known to be confined to plasma membranes. Many species show clearly demonstrable activity in bile canaliculi. However in the human liver canalicular activity is only occasionally encountered, and if so weak and focal. The rarely occurring extensive canalicular network observed in human liver tissue stained for alkaline phosphatase (9) might reflect activity of an isoenzyme of non-specific alkaline phosphatase possibly identical with the common, sinusoidal enzyme, or a more specific phosphatase. The canalicular activity might, alternatively be a manifestation of a genetically determined, normal variant of phosphatase, or of an increased activity due to an unknown activator The increased acti

34*

vity could also be explained by activation of a preformed proenzyme, or by decreased degradation or increased synthesis of enzyme protein. The enzyme might be of hepatic or extra hepatic origin.

In an endeavour to shed light on some of these possibilities human biopsy specimens with intense and extensive canalicular alkaline phosphatase activity were examined for other enzymes.

MATERIAL AND METHODS

The material consisted of all the 56 human liver biopsy specimens with an extensive canalicular activity of alkaline phosphatase but normal or almost normal histological appearance as judged from sections stained with routine methods (haematoxylin-erythrosin, van Gieson stain, periodic acid-Schiff (= PAS) according to Mac Manus before and after diastase digestion, Prussian blue, reticulin stain) found in a material of 620 specimens (Fig. 1). Specimens with an extensive canalicular phosphatase reaction from tissues showing cirrhosis, obstructive jaundice, hepatitis or malignant disease were not included because advanced changes in histochemical patterns of enzymes occur in these conditions irrespective of the presence or absence of canalicular alkaline phosphatase activity. Sections were stained for succinic and lactic dehydrogenases, monoamine oxidase, glucose-6-phosphatase, non-specific esterase, beta-glucuronidase, acid phosphatase, adenosine triphosphatase, leucine aminopeptidase, gamma-glutamyl transferase (1 2). However some of the 56 specimens were not stained with all the methods and staining was not always successful.

RESULTS

Sections stained for *succinic and lactic dehydrogenases monoamine oxidase glucose-6-phosphatase non specific esterase* showed nothing remarkable.

Staining for *adenosine triphosphatase* regularly resulted in visualization of thin canalicular structures and somewhat diffuse sinusoids. The canalicular reaction was never abnormally strong (Fig 2). Out of 53 liver biopsy specimens stained for *gamma-glutamyl transferase* 22 showed a canalicular network throughout the hepatic lobules, while the remaining 31 exhibited a normal appearance with scanty positive canaliculi around the portal zone and activity in bile duct epithelium (Fig 3). 49 of the liver biopsy specimens were stained for *leucyl aminopeptidase* and 12 of these showed an abnormal pattern with thickened and unusually knotty canaliculi, while the sinusoidal activity was markedly reduced (Fig 4). In normal human liver sinusoidal activity is demonstrable throughout the lobules. It is stronger in the peripheral third of the lobules. The activity is fairly uniform in the canaliculi.

The lysosomal enzymes, *acid phosphatase* and *beta-glucuronidase* normally show pericanalicular activity in hepatocytes. Acid phosphatase activity is demonstrable also in Kupffer cells. In 20 of 31 liver specimens examined beta-glucuronidase activity was clearly increased with scattered positive staining of lysosomal granules in the cytoplasm of the hepatocytes, with a tendency to crowding in the pericanalicular region. A similar pattern of activity was observed regarding acid phosphatase in 26 of 39 specimens examined. Most of these specimens also contained an increased number of acid phosphatase-positive Kupffer cells (Fig 5).

The number of the aforementioned enzymatic changes in a given specimen could vary from nil to all.

DISCUSSION

Judging from the present findings, a canalicular appearance of alkaline phosphatase activity in human liver biopsy specimens is

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Fig 1 Alkaline phosphatase reaction. *a*, Normal human liver (T 182/68) with activity in sinusoids centrolobularly (upper part) and periportally (lower part). *b* Human liver (T.6953/72) with canalicular alkaline phosphatase activity throughout the lobules. Portal zone down to the right, some positivity of sinusoids near the portal region. The nuclei are counterstained. × 180.

Fig 2 Adenosine triphosphatase reaction. *a* From a normal human liver (T 9656/72). *b* From a liver with canalicular alkaline phosphatase activity (T.6953/72). Similar activity of sinusoids and canaliculi in both specimens. Portal zones at bottom. No nuclear counterstaining. × 180.


1A
1B
2A
2B


3A
3B
4A
4B



Fig 3 Acid phosphatase reaction. a. From a normal human liver (T 182/68) Weak staining along canaliculi and scattered positive Kupffer cells. The intensity of the stain is very weak and is hard to reproduce photographically b From a human liver (T.9480/72) with increased reaction product along canaliculi and occurrence of more numerous positive Kupffer cells than normally. Same specimen as Fig. 4b. × 180.

Fig 2 Gamma-glutamyl transferase reaction. a. From normal human liver (T 182/68) with negative reaction. Some positive canaliculi near the portal zones are not visualized in the photomicrograph. b From a case (T 19552/72) with canalicular alkaline phosphatase activity with simultaneously increased canalicular activity of all the parenchyma in gamma-glutamyl transferase staining. The nuclei are counterstained. × 180.

Fig 4 Leucyl aminopeptidase reaction. a. Normal human liver (T.9616/72) with activity in sinusoids as well as canaliculi. b Human liver (T.9480/72) with mainly canalicular activity From a case with canalicular activity of alkaline phosphatase. In both *pictures portal zones at bottom with activity* in bile duct epithelium. The nuclei are counterstained. × 450.

often associated with a change in the patterns of other enzymes in the plasma membranes as well as with an increase in lysosomal enzyme activities, but unassociated with changes in enzymes in the cytosol, endoplasmic reticulum or mitochondria. An exception, however was adenosine triphosphatase, whose pattern was invariably normal also in the canaliculi. Thus, the proven alkaline phosphatase activity of canalicular microvilli does not seem to be identical with that of an adenosine triphosphatase. As for the rest of the plasma membrane markers studied, alkaline phosphatase and gamma-glutamyl transferase are known to increase in activity in rat liver tissue after administration of such substances as phenobarbitone and ethanol which induce the hydroxylating system of the

endoplasmic reticulum (11) Barbital anaesthesia *on a single occasion* has been shown to result in a marked increase in canalicular alkaline phosphatase activity for at least one week in the dog (3) We found an increase of canalicular activity of alkaline phosphatase and gamma-glutamyl transferase in human liver biopsy specimens after consumption of ethanol (7 8) The connection between the induction of the hydroxylating system of the endoplasmic reticulum and the canalicular increase in the above-mentioned enzymes is obscure. So far *nothing is known* about the ultrastructure of liver specimens *exhibiting* canalicular alkaline phosphatase activity The pattern of glucose-6-phosphatase (marker for endoplasmic reticulum) was normal in the present biopsy specimen However plasma membranes are produced by the internal membrane system of the cells. The variation in enzyme composition of the plasma membrane might reflect a specific or non specific process secondary to the induction phenomenon of the hydroxylating system in the endoplasmic reticulum. The high *frequency of canalicular alkaline phosphatase* activity in alpha 1-antitrypsin deposits is interesting in this connection, since the Z gene seems to cause accumulation of the antitrypsin protein in the smooth endoplasmic reticulum (6) The canalicular activity of alkaline phosphatase as well as gamma glutamyl transferase occurs in all parts of the hepatic lobules, and does not seem to be directly related to sites of accumulated protein.

It is known that hepatic lysosomal enzyme activities are increased in the presence of malignant diseases within or outside the liver (12 13) The histochemical findings in this study lend support to the biochemical observations. But they also showed that the activities of acid phosphatase and/or beta glucuronidase may be increased in connection *with canalicular alkaline phosphatase* also in the absence of malignant disease. The possibility *of a connection between the enzyme changes* of plasma membranes and lysosomes can at present be only *conjectural*.

Judging from the findings, the canalicular alkaline phosphatase is not an adenosine triphosphatase. The changes in other plasma membrane enzymes agree best with the assumption of a true increase in enzyme activity which seems to be connected with the so-called induction phenomenon of the hydroxylating system of the smooth endoplasmic reticulum.—Malignant tumour tissue is known to produce many different proteins, including an alkaline phosphatase similar to the placental isoenzyme (5) However according to the modern consensus of opinion the enzyme proteins are destroyed mainly in the reticuloendothelial system. The rather large enzyme molecule is presumably not secreted in the bile (4) The increased canalicular activity of the three plasma membrane enzymes studied are therefore most probably of hepatic origin.

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PREFERENTIAL INCORPORATION OF ³H-DEOXYCYTIDINE INTO CHICKEN BURSAL CELLS

Comparison of Radiolabelled Nucleosides as Lymphoid Cell Markers in Chickens and Mice

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Bäck, R. & Linna, T J Preferential incorporation of ³H-deoxycytidine into chicken bursal cells. Comparison of radiolabelled nucleosides as lymphoid cell markers in chickens and mice. Acta path. microbiol. scand. Sect. A, 83 534–538, 1975.

³H-deoxycytidine, ³H thymidine and ¹²⁵I-deoxyuridine were injected intravenously into different groups of chickens and mice and lymphoid organs and intestine of the animals sampled one hour later Incorporation of the isotopes into proliferating cells was evaluated after nucleic acid extraction and determination of specific activity of the isotopes in counts per minute per microgram of DNA (cpm/µg DNA) Labelled deoxycytidine was incorporated much better into bursal cells than the other isotopes, indicating that this isotope may preferentially label bursal lymphocytes, or a subpopulation of them.

Key words Bursal cells chicken ³H-deoxycytidine incorporation

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Radioactive thymidine has been widely used for studies of lymphoid cell kinetics because of its specific incorporation into DNA of proliferating cells (9 22) However ³H thymidine incorporation into central lymphoid organs, such as the thymus and the avian bursa of Fabricius, is low compared to that of peripheral lymphoid organs where cell proliferation is less extensive (5 15) The mechanism behind this discrepancy is not apparent but may at least in the case of the thymus, depend on the thymidine pool in the organ (7) Differences in labelling patterns of lymphocyte populations have also been reported to occur if other nucleic acid precursors are used. Thus, in rats, ³H-uridine labels thymus-derived (T) lymphocytes better than bone marrow-derived (B) lymphocytes (14)

Some recent data have suggested that thymus lymphocytes would utilize ³H-deoxycytidine better than ³H-thymidine, and that ³H deoxycytidine would be more effectively incorporated into thymus lymphocytes than into lymphocytes in peripheral lymphoid organs in mice (19) The specific activity of label was found to be higher in thymic DNA than in splenic DNA after labelling of rats with ³H deoxycytidine (13) These differences may be related to differences in pathways of formation of DNA thymine in thymus cells and pe-

ripheral lymphocytes (19). The presence of terminal deoxynucleotidyl transferase exclusively on thymus cells in several species (4) indicates that the thymus may use unique pathways in its DNA metabolism.

In the present investigation, ^{3}H-deoxycytidine, ^{125}I-deoxyuridine and ^{3}H-thymidine were injected intravenously into chickens and mice and the specific activity (cpm/µg DNA) in different lymphoid organs was calculated. The main purpose of this study was to obtain information whether any of these nucleosides would be preferentially incorporated into central lymphoid organs and consequently be particularly suitable for quantitative studies of central lymphoid organ cell kinetics.

MATERIALS AND METHODS

Nine-day-old random-bred White Leghorn (Babcock B-300) male chickens from a local vendor weight 90–177 grams, and young male mice of the CBA strain from the colony of the Department of Histology, University of Uppsala, weight 14–22 grams, were used. They were matched by weight into 3 groups, each consisting of 10–12 animals of each species. These groups of animals were injected intravenously with ^{3}H-deoxycytidine, ^{125}I-deoxyuridine and ^{3}H-thymidine respectively. All animals received 0.5 µCi per gram of body weight. The specific activities of the isotopes were the following: ^{3}H-thymidine, 6.7 Ci/mM; ^{3}H-deoxycytidine 15–30 Ci/mM; ^{125}I-deoxyuridine, 20–30 Ci/mM. The isotopes were obtained from New England Nuclear Corporation, Boston, Mass., USA.

One hour after labelling, the animals were sacrificed. In the chickens, the thymus, the bursa of Fabricius, the spleen and upper small intestine were dissected, while the thymus, the spleen, mesenteric lymph nodes and upper small intestine were sampled in the mice. The organs were immediately immersed into 5 ml of cold 5 per cent trichloroacetic acid and frozen at −20° C. The extraction of nucleic acids was performed using a modified Schneider technique (18). The DNA concentrations were measured according to Burton's modification (3) of the diphenylamine reaction. The tritium activity was determined in a Packard Tri-Carb scintillation counting system and the ^{125}I activity in a Packard gamma counter. The specific activity (spec. act.) of label in the different organs was calculated as counts per minute per µg DNA.

RESULTS AND DISCUSSION

In Tables 1 and 2 the mean specific activity (spec. act.) of each organ and its standard error are given. Since the mean specific activities of the different DNA precursors used

TABLE 1 *Specific Activity (Means ± S.E.M.) in Different Organs of 9-day-old Chickens Pulse-Labelled Intravenously with ^{3}H-thymidine, ^{125}I-deoxyuridine and ^{3}H-deoxycytidine Respectively*

Tissue studied	^{3}H thymidine	^{125}I-deoxyuridine	^{3}H-deoxycytidine
Thymus	19 ± 1	70 ± 6	12 ± 1
Bursa of Fabricius	76 ± 4	240 ± 21	78 ± 4
Spleen	123 ± 9	535 ± 60	63 ± 4
Intestine	124 ± 7	349 ± 26	56 ± 4

Specific activity = cpm/µg DNA.

TABLE 2 *Specific Activity* (Means ± S.E.M.) Value of Different Organs of Mice Pulse-Labelled Intravenously with ^{3}H-thymidine, ^{125}I-deoxyuridine and ^{3}H-deoxycytidine Respectively*

Tissue studied	^{3}H-thymidine	^{125}I-deoxyuridine	^{3}H-deoxycytidine
Thymus	8 ± 1	42 ± 3	8 ± 1
Mesenterial lymph nodes	24 ± 2	68 ± 4	12 ± 1
Spleen	38 ± 5	152 ± 23	38 ± 2
Intestine	75 ± 7	256 ± 10	35 ± 2

*Specific activity = cpm/µg DNA.

TABLE 3 *Percentual Activity (Means ± S.E.M.) in Different Organs of 9-day-old Chickens Pulse-Labelled Intravenously with ^{3}H-thymidine, ^{125}I-deoxyuridine and ^{3}H-deoxycytidine Respectively*

Tissue studied	^{3}H thymidine	^{125}I-deoxyuridine	H-deoxycytidine
Thymus	5 ± 1	6 ± 1	6 ± 1
Bursa of Fabricius	23 ± 1	20 ± 1	36 ± 1
Spleen	36 ± 2	45 ± 1	31 ± 1
Intestine	36 ± 1	29 ± 2	27 ± 1

$$\text{* Percentual activity} = \frac{\text{specific activity of an organ} \times 100}{\text{the sum of the specific activities of all sampled organs of the same animal}}$$

TABLE 4 *Percentual Activity* (Means ± S.E.M.) in Different Organs of Mice Pulse-Labelled Intravenously with ^{3}H-thymidine, ^{125}I-deoxyuridine and ^{3}H-deoxycytidine Respectively*

Tissue studied	^{3}H-thymidine	^{125}I-deoxyuridine	^{3}H-deoxycytidine
Thymus	6 ± 1	8 ± 1	9 ± 1
Mesenterial lymph nodes	17 ± 1	13 ± 1	13 ± 1
Spleen	25 ± 2	28 ± 3	41 ± 1
Intestine	52 ± 2	51 ± 3	38 ± 1

$$\text{Percentual activity} = \frac{\text{specific activity of an organ} \times 100}{\text{the sum of the specific activities of all sampled organs of the same animal}}$$

were of different magnitudes, the figures were normalized by calculating "percentual activities" i.e., the per cent spec. act. which is found in a certain organ of an animal, of the sum of the spec. act. of all sampled organs of that animal (Tables 3 and 4). In addition, the ratio between isotope incorporation into a lymphoid organ and incorporation into the intestine of the same animal was calculated for each sample and animal. Since intestinal epithelium proliferates rapidly (17, 24), it can be assumed that the main contribution to spec. act. in this organ is derived from epithelial cells. Therefore, this ratio relates incorporation of isotope to that in a rapidly proliferating structure lacking significant amounts of organized lymphoid tissue.

The mean spec. act. values and their standard errors applying to the different organs of the chickens are given in Table 1. ^{3}H-thymidine incorporation showed the expected regional differences (1). Among the lymphoid organs, the spleen showed the highest spec. act. values while the thymic and the bursal incorporation was comparatively low. The spec. act. values of ^{125}I-deoxyuridine, a thymidine analogue (8), showed the same pattern of incorporation as ^{3}H thymidine, with low figures in the case of the rapidly proliferating central lymphoid organs. Both these precursors were significantly better incorporated into the bursa of Fabricius than into the thymus. ^{125}I-deoxyuridine seemed to be somewhat better incorporated into the spleen than ^{3}H thymidine. These findings are also reflected in the normalized values in Table 3.

In the ^{3}H-deoxycytidine-labelled chickens, the highest spec. act. was surprisingly obtained in the bursa of Fabricius, while the lowest figures recorded were those in the thymus. This finding can also be seen in the normalized values in Table 3, suggesting that ^{3}H-deoxycytidine may preferentially label chicken bursa cells. Similar results were obtained by the comparisons of ratios between lymphoid organs and in-

testine after application of the different nucleosides tested.

The spec. act. values and the normalized values applying to the different organs sampled in the mice are given in Tables 2 and 4. In the mouse, all the injected precursors gave the same regional differences as ³H-thymidine. This was the case whether specific activities, percentual activities or ratios between lymphoid organs and intestine were used for comparison. Thus, there was no evidence of a selective labelling of thymus lymphocytes with ³H-deoxycytidine, as has been reported from other laboratories (13, 19). However, data similar to those obtained by us have been reported by *Gerber et al.* (10).

It seems that only bursal cells, or a proliferating subpopulation of the latter, would have the capacity to incorporate ³H-deoxycytidine preferentially. The spec. act. of ³H-deoxycytidine in the spleen and in the thymus, organs known to contain significant numbers of bursa-derived cells (2, 6, 12, 25), are lower than that in the bursa. The spec. act. values applying to the thymus and the spleen are in the same range as those applying to the other nucleosides.

Since ³H-thymidine and ³H-deoxycytidine both are incorporated specifically into DNA, although over partly different pathways (15, 16, 20, 21), the contamination of the samples with labelled RNA seems unlikely, especially since the tissues were sampled one hour after labelling, before labelled catabolic products could be expected to influence the data.

Thus, the present investigation shows that ³H-deoxycytidine is preferentially incorporated into chicken bursa cells. Its incorporation into the bursa was even more efficient than incorporation into the spleen, while the incorporation into the thymus was small. The observation that the spec. act. of label was clearly higher in the bursa than in the thymus after incorporation of all precursors used in this study further stresses the fact that cell proliferation in the bursa of Fabricius is very rapid (1, 11, 23) and indicates that the deoxyribonucleotide pool may be smaller in the bursa than in the thymus. We were not able to identify a nucleoside with preferential incorporation into thymic lymphocytes.

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PARATHYROID MORPHOLOGY IN GERBILS AFTER THYROIDECTOMY AND CALCIUM ADMINISTRATION

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Boquist, L. & Fåhraeus, B. Parathyroid morphology in gerbils after thyroidectomy and calcium administration. Acta path. microbiol. scand. Sect. A, 83: 539–549, 1975.

Morphological studies of the parathyroid glands from adult Mongolian gerbils in which hypercalcaemia had been induced by thyroidectomy and calcium injections for 2–19 days were carried out. The potassium pyro-antimonate technique and subsequent X-ray analysis of the precipitates were used for ultrastructural localization of cations. Ultrastructurally, most (suppressed) chief cells exhibited a dense cytoplasm, medium-sized or large mitochondria, glycogen particles, inconspicuous endoplasmic reticulum and Golgi complex, calcium-containing precipitates mainly in mitochondria and nuclei, and sometimes also lysosomal bodies and accumulations of secretory granules which occasionally seemed to be discharged into cytoplasmic vacuoles. A few parenchymal cells showed a low cytoplasmic density, few organelles, and structurally altered mitochondria, occasionally with associated smooth-surfaced vacuoles. These cells possessed calcium-containing precipitates in mitochondria, smooth-surfaced vacuoles, and also diffusely in the cytosol. It is concluded that, in the main part of the parenchymal cells, the hypercalcaemia had resulted in a suppression primarily of the synthetic and later also of the secretory activity, and that the calcium-containing precipitation is different in the chief cell variants.

Key words: Parathyroid morphology; thyroidectomy; gerbils.

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In a preceding work (*Boquist* 1975) parathyroid morphology was studied in Mongolian gerbils subjected to repeated injections of calcium alone or calcium in combination with parathormone (PTH). This treatment was found to induce a transitory hypercalcaemia and cellular changes consistent with low functional activity. However, conforming to the findings in other works using other species, time intervals, and experimental models (*cf. Capen* 1971, *Roth* 1971), it was noted that it is difficult, probably impossible, to induce complete inactivity or atrophy in the parathyroid glands by hypercalcaemic treatment of otherwise normal animals. One cause of this may be the stimulatory effect of calcitonin upon PTH secretion (*Oldham et al.* 1971). In experiments on rats, calcitonin has been shown to protect against hypercalcaemia (*cf. Sorensen* 1970).

In experiments on hypercalcaemia, the influence of calcitonin *in vivo* could be eliminated by thyroidectomy at least in certain species. This operation has previously and on the same indication been performed by us

on pigs (*Boquist & Fåhraeus* 1975 a, b) The anatomical relationship between the thyroid and parathyroid glands of Mongolian gerbils was studied in a preceding work (*Boquist & Fåhraeus* 1975 c) Thyroid C-cells are known to exist in rodent species and in the Mongolian gerbil C-cells have been observed by us in the thyroid, but not in the parathyroids or other organs (unpublished results) Accordingly it was considered worth while if the preceding studies of the parathyroid glands of normal Mongolian gerbils (*Boquist & Fåhraeus* 1975 c) and non-operated gerbils subjected to hypercalcaemic treatment (*Boquist* 1975) were supplemented by the present investigation of animals subjected to thyroidectomy and hypercalcaemic treatment. As no essential difference between animals injected with calcium alone and animals injected with calcium and PTH was observed in the preceding study only calcium injections were used in the present study

In addition to routine procedures for electron microscopy the potassium pyro-antimonate technique originally described by Komnick (*Komnick* 1962, *Komnick & Komnick* 1963) and subsequent X ray analysis of the precipitates was used for ultrastructural localization of cations. This technique has, to the best of our knowledge, *not* been used previously in studies of parathyroid glands. It was hoped that it might be possible by this technique to localize the cations, *e.g.* calcium, in the parathyroid glands.

MATERIAL AND METHODS

Animals Experimental Procedures and Serum Calcium Determinations

Nine adult Mongolian gerbils of both sexes were used. They were kept under standard laboratory conditions and had free access to drinking water and a standard laboratory diet (*Boquist* 1975) Thyroidectomy was carried out under Nembutal® (Abbott AB, Stockholm, Sweden) anaesthesia through a midline incision with the aid of an operation microscope (Zeiss Operationsmikroskop I, Carl Zeiss, Oberkochen, W Germany) After identification of the parathyroid and thyroid glands and their vascular supply the thyroidal vessels were ligated and the thyroid lobes were freed and removed upon which the wound was closed. No animal died during or after the operation.

At 8 a.m. and 4 p.m. on the first postoperative day all animals received intraperitoneal injections of calcium (Kalcium-levulat, AKO Solna, Sweden) in a total daily dose of 0.8 g/kg body weight. Similar injections were then given every day for 2 5 6, 7 (2 animals) 8, 11 14 and 19 days.

Sixteen hours after the last injection, the animals were again anaesthetized with Nembutal® and a new midline incision was made through which the parathyroid glands could be identified and removed for microscopic examination. Subsequently the aorta and the inferior caval vein were cut and blood was collected for calcium determination performed by atomic absorption spectrophotometry (Unicam SP90B Unicam Instrument Ltd., Cambridge) The standard used was CaCO in 0.75 per cent EDTA-solution. The error of the method was <1 per cent. For technical reasons, serum calcium determinations were only carried out at sacrifica. The serum calcium concentration in normal gerbils observed in the preceding study (*Boquist & Fåhraeus* 1975 c) was used for comparison with that observed in the present study

Microscopic Procedures and X-ray Analysis

The left parathyroid gland from each animal was taken for transmission electron microscopic study It was fixed by immersion in 2.5 per cent glutaraldehyde in 0.34 M Veronal acetate buffer adjusted to pH 7 4 followed by post-fixation in 1 per cent osmium tetroxide and embedding in Epon 812. Toluidine-blue-stained, thick sections are used for light microscopic identification of suitable areas for the fine structural examinations. The thin sections were stained with uranyl acetate and lead citrate and were then viewed in a Siemens Elmiskop 101 electron microscope.

The right parathyroid gland from each animal was used for electron microscopic localization of cations, using a modification of the original potassium pyro-antimonate technique (*Komnick* 1962, *Komnick & Komnick* 1963) The specimens are fixed by immersion for 2 hours in a solution of 3 per cent glutaraldehyde and 2 per cent potassium pyro-antimonate adjusted to pH 7 4 with 0 01 N acetic acid Subsequently the specimens were rinsed in a solution of 2 per cent potassium pyro-anti-

Fig 1 Suppressed parathyroid chief cells showing irregular nuclei, moderate cytoplasmic density rather sparsely developed endoplasmic reticulum and Golgi complex, mitochondria of varying appearance, storage granules (S) lysosomal bodies (Ly) and straight membranes with a few microvilli. × 8,000





Fig 2 Portion of suppressed chief cell exhibiting a rather high cytoplasmic density free ribosomes, numerous secretory granules, and a storage granule into which secretory granules seem to be released. The storage granule membrane shows varying thickness. The cell membranes re straight. × 21,000.

monate nd 5 per cent sucrose at pH 7 4 followed by post-fixation for 1 hour in a solution of 1 per cent osmium tetroxid and 2 per cent potassium pyro-antimonate djusted to pH 7 4 with 0.01 N acetic acid. Subsequently the specimens were rinsed in 5 per cent sucrose and dehydrated in ethanol and propylene oxide. The embedding, preparation of thick and thin sections, and microscopic examination were as described above.

The specificity of the precipitate was investigated by X-ray analysis using an energy-dispersive system (EDAX, Chicago) combined w th a Jeol JSM U3 Scanning Electron Microscope. This analysis was carried out according to current principles at the Wallenberg Laboratory Uppsala, Sweden, on unstained thin sections, and also on 1.5 μ thick sections, prepared by the potassium pyro-antimonate technique

RESULTS

The *mean serum calcium* level of the operated animals was 14.6 ± 2.7 mg/100 ml whereas that of normal gerbils was 10 2 ± 0 6 mg/100 ml.

Light microscopic examination of the toluidine-blue stained thick sections showed in all animals a similar picture of mainly solid, sheets of chief cells most of which were of the dark variant, while a few were of the light variant Scattered cells with an intermediate cytoplasmic staining affinity were also recorded. The predominance of dark cells was most marked from days 6–7 and onwards. A few follicles with an amorphous content were observed

Electron microscopic examination disclosed solid sheets of parenchymal cells and a few follicles. The parenchymal cells were represented by chief cell ariants. No oxyphil cells or water-clear cells were encountered.

At all observation times, and especially from days 6 to 7 and onwards, most parenchymal cells exhibited rounded or irregular nuclei, rather straight cell membranes with some microvilli a moderate or high cyto-



Fig 3 Suppressed chief cell showing a dense cytoplasm with numerous lysosomal bodies (Ly) × 14 000.

plasmic density often numerous glycogen particles, lipoid bodies, and rather inconspicuous endoplasmic reticulum and Golgi complex (Fig 1) They were interpreted as suppressed chief cells and possessed a moderate or large number of medium-sized or large mitochondria, which were elongated oval or rounded, and had distinct cristae and rather dense matrix. In some suppressed chief cells, mainly at days 2 and 5 there were cytoplasmic accumulations of secretory granules with finely granular cores. Similar finely granular material was seen in some Golgi complexes and also in electron lucent cytoplasmic vacuoles into which secretory granules occasionally seemed to be discharged (Fig 2) From days 6 to 7 and onwards, lysosomal bodies of varying appearance were seen in the cytoplasm of many suppressed cells (Fig. 3)

Other less frequently occurring cells possessed a low cytoplasmic density inconspicuous organelles concerned with hormone synthesis and storage, and a varying number of mitochondria. Some mitochondria showed electron lucent areas, dilated external membranes, occasionally of an appearance of bulbous projections and electron lucent, smooth-surfaced vacuoles (Fig. 4) These changes were seen in mitochondria which otherwise were normal, without any signs of degeneration. Similar electron lucent vacuoles were also seen in the cytoplasm, without any connection with mitochondria.

There were also some cells with characteristics intermediate between those of the cell types described above as well as scattered cells with a rather prominent endoplasmic reticulum and Golgi complex, and a moderate cytoplasmic density

The pyro-antimonate-treated sections showed intracellular and intercellular (Fig. 5) electron dense precipitates. In the predominating, suppressed chief cells there were granular electron dense precipitates in the dense



Fig 2 Portion of suppressed chief cell exhibiting a rather high cytoplasmic density free ribosomes, numerous secretory granules, and a storage granule into which secretory granules seem to be released. The storage granule membrane shows varying thickness. The cell membranes are straight. × 21,000.

monate and 5 per cent sucrose at pH 7 4 followed by post-fixation for 1 hour in a solution of 1 per cent osmium tetroxide and 2 per cent potassium pyro-antimonate adjusted to pH 7 4 with 0 01 N acetic acid. Subsequently the specimens were rinsed in 5 per cent sucrose and dehydrated in ethanol and propylene oxide. The embedding, preparation of thick and thin sections, and microscopic examination were as described abov

The specificity of the precipitate was investigated by X-ray analysis using an energy-dispersive system (EDAX, Chicago) combined with a Jeol JSM U3 Scanning Electron Microscope. This analysis was carried out according to current principles at the Wallenberg Laboratory Uppsala, Sweden, on unstained thin sections, and also on 1.5 μ thick sections, prepared by the potassium pyro-antimonate technique.

RESULTS

The *mean serum calcium* level of the operated animals was 14 6 ± 2.7 mg/100 ml, whereas that of normal gerbils was 10.2 ± 0 6 mg/100 ml.

Light microscopic examination of the toluidine-blue-stained thick sections showed in all animals a similar picture of mainly solid sheets of chief cells most of which were of the dark variant while a few were of the light variant Scattered cells with an intermediate cytoplasmic staining affinity were also recorded. The predominance of dark cells was most marked from days 6-7 and onwards. A few follicles with an amorphous content were observed.

Electron microscopic examination disclosed solid sheets of parenchymal cells and a few follicles. The parenchymal cells were represented by chief cell variants. No oxyphil cells or water-clear cells were encountered.

At all observation times, and especially from days 6 to 7 and onwards, most parenchymal cells exhibited rounded or irregular nuclei, rather straight cell membranes with some microvilli a moderate or high cyto-



Fig 5 Portion of parathyroid cells showing a moderate cytoplasmic density and free ribosomes. Dense precipitates are seen in the intercellular space. Pyro-antimonate fixation. × 30,000.

of calcitonin upon the serum calcium level and agrees with the findings in studies of rats subjected to thyroidectomy thyroparathyroidectomy or parathyroidectomy (*Talmage et al* 1965 *Gittes & Irvin* 1966 *Sorensen* 1970, *Harper & Toverud* 1973)

The synthetic and secretory activity of the parathyroid glands is regulated by the ambient calcium concentration there is an inverse relationship between the release of PTH and changes of the calcium level (*cf Sherwood et al.* 1971) A similar although not identical, role is played by magnesium on parathyroid activity (*f Sherwood et al.* 1970 1971) Effects upon the hormone secretion (*Blum et al* 1974) and energy metabolism (*Hennion & Hamberger* 1973) have been found even after small or moderate changes in the concentration of these cations.

In the present study the findings in most parenchymal cells of structural signs of low activity in the organelles concerned with hormone synthesis and storage are consistent with a suppressive role of existing hypercalcaemia on parathyroid activity In addition, the cells with such signs of low activity were usually characterized by a moderate or high cytoplasmic density and sometimes by enlarged mitochondria. Cells presenting these characteristics were interpreted as functionally suppressed cells and, since we believe in the existence of a functional cyclus in the parathyroid cells (*Capen* 1971 *Roth* 1971 *Altenähr* 1972 *Boquist* 1975) the suppressed cells are thought to be in an inactive stage of the cyclus, as regards synthesis and release of hormone.

In some cells which otherwise appeared suppressed there were numerous secretory granules, conforming to the findings in suppressed glands of other species (*Capen* 1971 *Black et al.* 1973 *Nunez et al* 1974) This agrees with the opinion that the inhibition of the secretion precedes the inhibition of the synthesis of hormone in parathyroid cells (*Roth & Raisz* 1966 *Oldham et al.* 1971) The suppressed cells also possessed cytoplasmic vacuoles with a finely granular content

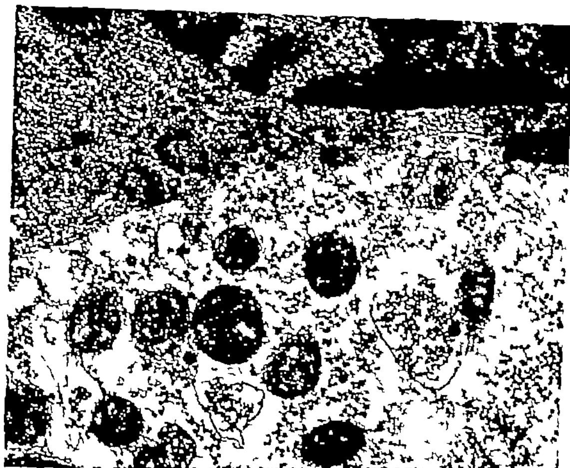


Fig 6 Portion of suppressed chief cell (above) and one chief cell with a low density showing electron dense precipitates in smooth-surfaced, electron lucent vacuoles connected with mitochondria, and in rough-surfaced vacuoles (arrows) Pyro-antimonate fixation × 25,000

and, occasionally with signs of discharge of secretory granules into these vacuoles. In other studies such vacuoles have been interpreted as abnormal secretory granules (*Capen* 1971) osmiophilic bodies containing "unstabilized" hormone (*Rohr & Krassig* 1968) or secretory granules with stored mature hormone (*Altenähr* 1970) We have interpreted these vacuoles as storage granules to appear in suppressed cells when there is no immediate need for hormone secretion. We also believe that these storage granules can be released if the calcium concentration is decreased. Possibly this release takes place under interaction of lytic enzymes (*Stoeckel & Porte* 1966, *Hara & Nagatsu* 1968, *Rohr & Krassig* 1968) If the hypercalcaemia persists, at least for some time the storage granules are believed to undergo lysosomal digestion in a way similar to that observed among secretory granules in the endocrine pancreas (*Boquist* 1970) The presence in the suppressed cells, mainly at the later observation times, of lysosomal and lipoid bodies, and the decreased frequency of cells with accumulations of secretory granules at these observation times, support this opinion.

As in the previous study of non-operated animals subjected to hypercalcaemic treatment (*Boquist* 1975) it was not possible either in the present study to induce complete cellular inactivity although the effect of calcitonin most probably had been eliminated. The reason may be the existence of a functional cyclus and accordingly at least some cells would then be active even in suppressed



Fig 7 Parathyroid area showing portion of one chief cell of low density with few organelles and electron dense precipitates diffusely in the cytosol and portion of two cells of moderate density with more prominent organelles. The small, dense particles in the cytoplasm of the latter cells represent ribosomes. Intercellular precipitates are also present. Pyro-antimonate fixation. × 19,000.

glands. This would be consistent with the view that there is a basal release of PTH which cannot be suppressed by experimental means (*Cutes & Radde* 1966, *Roth & Raisz* 1966). Other factors, *e.g.* magnesium, adrenalin and glucagon might also play a role in this respect.

The data obtained in the present study do not allow any definite appraisal of the functional activity of the parenchymal cells with a low cytoplasmic density or of the correlation between a possible functional activity and the occurrence of glycogen, lipoid bodies, appearance of cell membranes as well as size of intercellular spaces.

The results of the pyro-antimonate-technique should be interpreted with some caution since there is considerable evidence that the specificity of this technique is limited (*Shina et al* 1970, *Torack & LaValle* 1970). X-ray analysis of the precipitates has been recommended because of this lack of specificity (*Mizuhira* 1973). In the present study such analysis disclosed the presence of calcium both in the intercellular and intracellular precipitates. The finding of calcium-containing precipitates in the mitochondria is not unexpected in view of the well-known calcium-binding capacity of these organelles in other tissues (*cf. Lehninger* 1970). As a working hypothesis, it is believed that one function of mitochondria in parathyroid cells may be storage and/or buffering of intracellular calcium which may occur in a hypercalcaemic

environment, and that the observed difference in calcium-containing precipitation in suppressed cells and in those with a low cytoplasmic density might be of functional significance.

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CARCINOGENICITY IN MICE OF SOME FATTY ACID METHYL ESTERS

2 Peroral and Subcutaneous Application

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Kiær H. W., Glavind, J & Arffmann, E. Carcinogenicity in mice of some fatty acid methyl esters. 2 Peroral and subcutaneous application. Acta path. microbiol. scand. Sect. A, 83 550–558, 1975

Two fatty acid methyl esters, methyl oleate and methyl 1 -oxo-t ans-10-octadecenoate have been tested for carcinogenicity by oral and subcutaneous administration in ST/a mice of both sexes A positive effect of methyl oleate could not be assessed, while the results pointed to a promoter effect of methyl oxo-octadecenoate. Given in the diet, this compound increased the incidence and number of forestomach papillomas within 83 weeks after initiation by 4-nitroquinoline 1-oxide. Repeated injections of methyl oxo-octadecenoate in the inguinal area resulted in 2 local sarcomas in a group of 20 females which had previously received skin initiation by 7 12-dimethylbenz[a]anthracene in the neck In no other animal group did sarcomas appear at this location within the observation period of 2 years. An influence by the injected methyl esters on the initiated skin carcinogenesis was possibly but weakly present The need for more extensive experiments is stressed, especially with a view to the possible carcinogenic hazards involved in dietary intake of oxygen-containing derivatives of oleic acid

Key words Carcinogenicity fatty acid methyl esters mice

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Previous studies of the carcinogenicity of fatty acid methyl esters applied to the skin of mice have shown a weak effect of oleate hydroxyoctadecadienoate and 12-oxo-trans 10-octadecenoate. The methyl esters of the fatty acids acted essentially as promoters (Arffmann & Glavind 1974)

Carcinogenicity testing should comprise the use of more than one route of administration, and the preferred route should be that suspected to operate in man (Berenblum 1969) As regards fatty acids, this route is by mouth.

A promoter effect might be disclosed by initially feeding a gastric carcinogen in a subeffective dose The induction of carcinogenesis of the gastro-intestinal tract by two-stage techniques has been reported previously (Berenblum & Haran-Ghera 1957 Peirce 1961 Narisawa et al 1974) The work of Sugimura et al. (1969 ha furnished a method to be used for the experimental induction of gastric adenocarcinomas t a high incidence in rats. Facilities for the preparation of methyl 12 oxo-trans 10-octadecenoate in a scale sufficiently large for a long term feeding experi-

ment in rats were, however not at our disposal. The only alternative was to use mice although the experimental production of tumours in the glandular stomach is much more difficult in these animals than in rats. The 4-nitroquinoline 1-oxide seemed to be the most adequate substance to be chosen as inducer although its weak effect on the glandular stomach presents a disadvantage. It has a strong effect on the forestomach and is also a potent skin carcinogen (*Kinosita* 1969 *Mori* 1969) It has been designated as a carcinogen with multiple target organs (*Konishi et al.* 1974) 4-Nitroquinoline 1 oxide has not previously been used as initiator in two-stage carcinogenesis of the stomach.

As subcutaneous application of a test chemical is a highly sensitive method which discloses the activity of most known carcinogens (*Hueper & Conway* 1964) this way of testing was added as a third mode of application. A possible promoter effect was registered as an enhancement of skin carcinogenesis initiated by 7 12-dimethylbenz[a]anthracene and, on the other hand, the skin treatment might stimulate subcutaneous tumour formation and incidentally a systemic effect in the mice.

Methyl 12-oxo-*trans* 10-octadecenoate, the fatty acid derivative found to be most active in the previous experiment, together with the ester of native oleic acid, have now been tested by peroral and subcutaneous application.

MATERIALS AND METHODS

Substances. The preparation of methyl 12-oxo-*trans*-10-octadecenoate (MOO) has been described previously (*Arffmann & Glavind* 1974)

Methyl oleate (MO) was from Fluka (Oelsäure-methylester (natürlich) purum. ≥98 per cent) and was further purified by column chromatography on alumina (Aluminiumoxyd für die Säulenchromatographie neutral, Merck) A solution in petrol ether was applied to the column. The methyl oleate was eluted with petrol ether ethyl ether (95 5)

For feeding, the fatty acid methyl esters were mixed with the powdered stock diet in the proportion 3 75 mg ester per g food In order to facilitate the manufacture of tablets of the mixture the following quantities of adjuvants per g food were added microcrystalline cellulose (Avicel (FMC Corporation, Newark, Delaware, USA)) 230 mg sodium carboxymethyl cellulose (Ph. Nord.) 15 mg magnesium stearate (Ph. Nord.) 10 mg talc (Ph. Nord.) 14 mg silica (silicii oxidum (DAK preparater 1963)) 26 mg. New tablets were made regularly during the experiment. It was observed that each mouse consumed on an average 4 tablets of 1,315 g per day corresponding to a daily intake of 15 mg ester

Some of the tablets to remain after completion of the feeding treatment were analysed and the recovery of the methyl oxo-octadecenoate was determined. The tablets were extracted with chloroform—methanol (2 1) The extract was evaporated and fractionated by column chromatography on silicagel (Kieselgel HR, Merck) The fraction obtained by elution with petrol ether—ethyl ether (90 10) was evaporated and dissolved in hexane (n-Hexan für die Spektroskopie, Merck) and examined in a Beckman Spectrophotometer DU A maximum was observed at 224 nm, and it could be calculated from the optical density that 62 per cent of the ester originally added to the tablets had been recovered.

For subcutaneous injection, the test substances were dissolved in triolein (British Drug House) which had been purified by chromatography on alumina (Aluminiumoxyd aktiv neutral, für die Säulen-Chromatographie, Merck) The triolein was eluted with petrol ether—ethyl ether (80 20) Preliminary experiments using injections of 5 and 10 per cent solutions (v/v) for 2 months showed that both concentrations were tolerated without local necrosis.

The 4-nitroquinoline 1-oxide (NQO) was from Fluka (4-Nitrochinolin-N-oxid (purum)) A 0.1 per cent solution (w/v) in 10 per cent ethanol (w/w) was prepared following the procedure of *Kinosita* (1969) A small part of the substance could not be dissolved in spite of heating and vigorous stirring

The 7 12-dimethylbenz[a]anthracene (DMBA) was from Sigma Chemical Company It was dissolved in acetone (pro analysi, Merck) to make 0.1 per cent solution (w/v)

Animals Male and female inbred ST/a mice were used. Housing and care were as described in our previous paper They were fed a pelleted stock diet (Rostock mixture Korn- og Foderstofkompagniet, Copenhagen)

The animals employed in the injection experiment were 6½ to 8 weeks old when the initial skin application of DMBA was made. Males then weighed about 16–17 g, females about 13–14 g. The feeding experiment was started when the animals were 8–10 weeks old, males then weighing 22–27 g, and females 16–19 g. The use of larger animals facilitated the tube feeding

As far as possible the mice were randomly dis-

tributed over the various experimental groups, but males were distributed as littermates to reduce fighting.

Feeding experiments. Initiating was performed by giving the solution of NQO through stomach tube (Acufirm needle nr 1464 a) three times a week for two weeks. Each intubation was done in the morning and had been preceded by 17 hours of fasting. The initial dose was 0.1 mg, the following doses were 0.2 mg each. The total dose of 1.1 mg NQO per mouse was presumed to be subeffective as the effective doses used by *Kinosita* (1969) were about 5 to 8 times higher.

Each experimental group consisted of 15 male and 15 female mice. One group (F 1) was left untreated. A second group (F 3) was given initiation and no further treatment. Two groups were treated with initiator and, 9 days after the last dose, transferred to the tabletted diets containing MO (Group F 4) and MOO (Group F 5) respectively. Finally the same diet as in Group F 5 was given to a group without previous treatment with initiator (Group F 2). After 300 days, the animals in the groups given fatty acid methyl ester were transferred to the stock diet.

Positive controls were not run as a higher concentration of NQO in ethanol could not be achieved and, considering the difficulties involved in tube feeding of mice, the application of larger single volumes or an increase of the number of applications was not found practicable.

The animals were inspected and weighed once every two weeks.

Surviving animals were killed after 84 weeks. Autopsy was performed on all mice. The stomach, spleen, left kidney, liver, lungs and heart were routinely examined histologically.

Subcutaneous application. For initiation, DMBA was applied in a subeffective dose (50 µg) to the skin as described in our previous paper (*Arffmann & Glavind* 1974). This was the only treatment of the control group I 6. Groups I 7 and I 9 were given weekly subcutaneous injections of 10 per cent solutions in triolein of MO and MOO respectively. The injections were given in the right groin. The volume was 0.1 ml and injection started 1 week after the initiation. For comparison, the groups I 2 and I 4 were injected with MO and MOO in the same doses as groups I 7 and I 9 but without previous initiation. Group I 1 was left as an untreated control group. All the groups comprised 15 male and 15 female mice.

After weekly applications for about three months, necrosis appeared at the injection site, most severe after MOO. After 16 weeks, the injections were interrupted. They were resumed 16 weeks later when necrosis had disappeared, but now only 5 per cent solutions and a volume of 0.05 ml per injection were given. After ten weekly applications of the small dose, a total of 26 injections had been given, furnishing a total amount of 0.185 ml of fatty acid methyl ester per mouse.

Having seen the necrotic effect of the larger doses of the esters we decided to enlarge the experimental material with four additional treatment groups given smaller doses. Each of the additional groups comprised 20 female mice which were given weekly doses of 0.05 ml of the 10 per cent solutions of the fatty acid methyl esters. Two of the groups, I 3 and I 8, received MO and two groups, I 5 and I 10, were given MOO. The groups I 8 and I 10 had been initiated with DMBA one week before the first injection. Necrosis appeared in the groin, even after the smaller doses, and the treatment was finally discontinued after 5 injections. A total amount of 0.025 ml of fatty acid methyl ester per mouse had been given.

The animals were inspected weekly and regularly weighed. Skin papillomas were counted in the cumulative totals when at least 1 mm in diameter and observed at 3 successive inspections. Palpable masses in the inguinal area were recorded. Animals with large tumours were sacrificed. Surviving animals were killed after 2 years. Autopsy was performed on all mice. Macroscopic lesions were processed for histological study.

RESULTS

Carcinogenicity testing of methyl oleate and methyl 12-oxo-*trans* 10-octadecenoate by *oral administration* gave the results shown in Table 1.

Squamous cell papillomas of the forestomach were the most frequent tumours (Fig. 1). They were single or multiple, often situated near the limiting ridge and might be accompanied by a more extensive papillomatosis. Papillomatosis alone and papillomas with diameters less than 1 mm were not counted. The first papillomas were observed in the animals that died between 57 and 60 week.

One squamous cell carcinoma was observed; it appeared in the forestomach of a male mouse which had been treated only with initiator and which died after 74 weeks. The tumour occupied the whole forestomach and invaded the glandular stomach and neighbouring organs. There were metastatic deposits in the regional lymph nodes.

Adenocarcinomas of the glandular stomach were not seen. In three mice which carried also from 2 to 6 papillomas of the fore-

TABLE 1 *Tumour Induction in ST/a Mice by Peroral Application of Some Fatty Acid Methyl Esters as Promoters C mulative Results after 84 W ks*

Group	Treatment		No. of mice	Survivors at		No. of mice with forestomach papilloma(s)	Total no. of forestomach papillomas	Mice with other tumorous conditions of the stomach-oesophagus	No. of mice with lymphoma
	Initiator	Promoter		57 weeks	84 weeks				
F 1	None	None	30	20	9	0	0	0	2
F 2	None	MOO‖	30	24	11	0	0	0	3
F 3	4-NQO†	None	30	19	5	4	4	1 Squamous cell carcinoma of the forestomach.	2
F 4	4-NQO†	MO‡	30	22	15	5	8	1 CIS§ of the glandular stomach. 1 Papilloma oesophagi.	2
F 5	4-NQO†	MOO‖	30	16	14	7	16	2 CIS§ of the glandular stomach.	3

Time of the first discovery of forestomach papilloma at autopsy
† 4-Nitroquinoline 1-oxide, 0 1 per cent (w/v) in 10 per cent ethanol, total dose 1 1 mg.
‖ Methyl 12-oxo-*trans*-10-octadecenoate, mixed in the stock diet to yield a daily dose of 15 mg per mouse, given for 300 days.
‡ Methyl oleate, mixed in the stock diet to yield a daily dose of 15 mg per mouse, given for 300 days.
§ Carcinoma in situ.

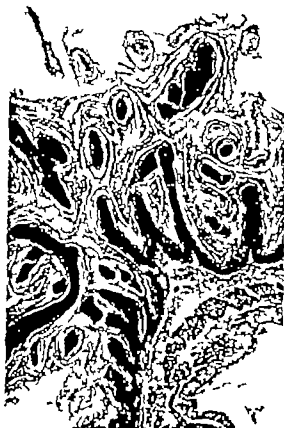


Fig 1 Squamous cell papilloma of the forestomach in a male mouse given initially 4-nitroquinoline 1-oxide by stomach tube and then methyl 12-oxo-*trans*-10-octadecenoate in the diet for 300 days (Group F 5) The animal died from pulmonary infection 139 days after cessation of the treatment (ca. 50 ×)

stomach, a small area of the glandular mucosa bordering the limiting ridge showed a glandular hyperplasia and epithelial atypia which could be designated as carcinoma *in situ* or mucosal carcinoma (*Mori* 1969) (Fig 2) The mice were from the groups treated with fatty acid methyl esters as promoters, and the lesions were not grossly detected.

The autopsies revealed no tumours in the intestines. The weight curves of the experimental animals did not differ from those of the controls.

The results of the *injection experiment* are seen in Table 2.

Benign skin tumours were papillomas. The malignant tumours were squamous cell carcinomas except for one sarcoma in group I 10

Two tumours appeared at the injection site in two mice in group I 10 after 215 and 539 days. The first mouse was sacrificed after 243 days, the second animal after 568 days. Histological examination showed sarcomas of the fibroblastic type. The first tumour was observed to surround closely the scattered remnants of the injected oily material (Fig. 3)

Malignant lymphomas appeared both in the feeding experiment and in the injection experiment. Most of these tumours were histiocytic neoplasms or plasmacytomas.

Other tumours of quite varied nature appeared especially in the lungs, the female genitals, the liver and the skin. There was no correlation with treatment, and the tumours are not shown in the tables.

Intercurrent deaths were mainly due to pulmonary infections in the form of purulent bronchitis, atelectases and bronchopneumonia.



Fig 2 Carcinoma *in situ* in small area of the glandular stomach bordering the limiting ridge Same mouse as in Fig 1 (ca. 125 ×)

TABLE 2 Tumour Induction in ST/a Mice by Subcutaneous Application of Some Fatty Acid Methyl Esters without or after Preceding Initiation of the Skin with 7,12 Dimethylbenz[a]anthracene: Cumulative Results after 2 Years

Group	Treatment: Initiator (dose/mouse)	Treatment: Promoter (dose/mouse)	No. of mice	Survivors †: 12 mths.	Survivors †: 24 mths.	Cumulative no. of mice with skin tumours: Benign	Cumulative no. of mice with skin tumours: Malignant	No. of days prior to first skin tumour	No. of mice with tumours at the injection site	No. of mice with lymphoma	No. of days preceding first death of animal with lymphoma
I 1	None	None	30	23	8	1	0	449	0	2	644
I 2	None	MO* 0.185 ml	30	22	8	4	0	450	0	3	655
I 3	None	MO§ 0.025 ml	20	20	15	1	0	706	0	3	542
I 4	None	MOO‖ 0.185 ml	30	23	9	1	0	641	0	3	646
I 5	None	MOO¶ 0.025 ml	20	17	15	2	0	320	0	0	—
I 6	DMBA† 50 μg	None	30	22	3	11	0	269	0	6	100
I 7	DMBA† 50 μg	MO* 0.185 ml	30	24	13	6	1	159	0	3	163
I 8	DMBA† 50 μg	MO§ 0.025 ml	20	19	11	3	1	331	0	3	578
I 9	DMBA† 50 μg	MOO‖ 0.185 ml	30	26	8	10	1	72	0	3	509
I 10	DMBA† 50 μg	MOO¶ 0.025 ml	20	18	5	5	1	189	2	2	401

† 7,12 Dimethylbenz[a]anthracene, 0.1 per cent (w/) in acetone, single application.
Methyl oleate, 10 per cent (v/v) in triolein 0.1 ml once weekly for 16 weeks, after a 16 weeks' interval without injections 5 per cent (v/v) in triolein 0.05 ml once weekly for 10 weeks.

§ Methyl oleate, 10 per cent (v/v) in triolein 0.05 ml once weekly for 5 weeks.

‖ Methyl 12-oxo-trans-10-octadecenoate, 10 per cent (/v) in triolein 0.1 ml once weekly for 16 weeks, after a 16 weeks' interval without injections 5 per cent (v/v) in triolein 0.05 ml once weekly for 10 weeks.

¶ Methyl 1 -oxo-trans-10-octadecenoate, 10 per cent (v/v) in triolein 0.05 ml once weekly for 5 weeks.

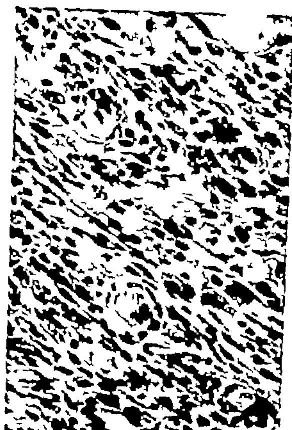


Fig 3 Inguinal fibrosarcoma in a female mouse receiving skin initiation by 7 12-dimethylbenz[a]anthracene and then subcutaneous injections in the groin of methyl 12-oxo-*trans*-10-octadecenoate (total dose 0.025 ml) (Group I 10) Remnants of the injected oily material are seen deposited in several small holes in the tissue. (ca. 400 ×)

DISCUSSION

Kinoshita (1969) reported a high incidence of papillomas in the forestomach of mice within the first 4 to 5 months of peroral administration of NQO A few months later squamous cell carcinomas appeared in up to 50 per cent of the animals. *Kinoshita* observed one adenoacanthoma of the glandular stomach among 14 mice and *Mori* (1969) observed one glandular carcinoma among 21 mice. The effective peroral dose of NQO in these experiments was 5 to 8 mg In their experimental study of oral cancer *Wallenius & Lekholm* (1973) observed that the forestomach in rats was sensitive to NQO

The total dose of NQO used by us was only 1.1 mg per mouse and yet, four mice out of an effective total of 19 in group F 3 developed a papilloma in the forestomach (effective total is number of mice surviving 37 week, the time of autoptic discovery of first stomach papilloma)

The ingestion of MO did not increase the relative number of tumour-bearing mice, but the total number of papillomas was increased.

A greater difference was seen, however in group F 5 in which the diet was supplemented with MOO The percentage of papilloma-bearing mice as well as the number of forestomach papillomas per animal was twice that seen in the control group F 3 The fact that the survival rate during the last year was higher in group F 5 than in F 3 may complicate the comparison between the two groups, but the tumour yield was also found to be higher in group F 5 than in group F 4 More exact intergroup comparisons, eliminating the different mortality patterns, might be performed by calculating the expected papilloma incidences for successive weeks and comparing them with the incidences actually observed. Such analysis would however require larger animal groups Moreover papillomas of the forestomach did not act as a cause of death and could accumulate in the surviving animals during a protracted latency period

As regards the papilloma-bearing mice in group F 5 three belonged in the group of 4 surviving males while the remaining 4 belonged in the group of 12 surviving females. Although the result could indicate an influence of sex on the tumour induction, the material is too small to allow any conclusion.

Early malignant changes in the glandular stomach were only seen in the two animal groups promoted by fatty acid methyl esters. The incidence was, however too low to allow definite conclusions. The appearance of one squamous cell carcinoma in group F 3 is probably an expression of the biological variation in sensitivity to NQO

The high intercurrent mortality in several animal groups was probably connected with climatic instability and the periodic appearance of epizootics in our newly established

animal quarters. An influence of the applied fatty acid esters on life span, as Table 1 might suggest, seems a less probable explanation. The added amounts of MO and MOO (0.375 per cent) are relatively small compared with the quantity of lipids previously present in the stock diet (2.9 per cent). *Kaunitz et al.* (1970, 1973) have studied the influence of the quality of fats on the life span of rats and found significant differences when different fats were fed. The causal relations were, however, very complicated and the material did not include fatty acid methyl esters.

The results of the feeding experiment suggest a promoting potency of MOO in gastric carcinogenesis. The occurrence of oxo-octadecenoate among the products formed by autoxidation of oleic esters recalls the observation that several anti-oxidants inhibit polycyclic hydrocarbon-induced carcinogenesis in the forestomach of the mouse (*Wattenberg* 1972). The negative results in Group F 2 shows that MOO did not act as a complete carcinogen. The incidence of lymphomas was not influenced by the treatment.

Swern et al. (1970) tested fatty acids and derivatives for carcinogenic activity by repeated subcutaneous injections in the inguinal area of female mice. Twenty-nine compounds were examined, and several hydroxy-, keto- and epoxy-derivatives and one peroxy-derivative induced sarcomas within 2 years. A positive result obtained by stearic acid, however, raised serious questions regarding the interpretation of the results. By way of conclusion, it did appear that "weak carcinogens for the subcutaneous tissue of mice occur among these classes of chemical compounds".

Some of the compounds were tested again in two co-operating laboratories where similar results were obtained (*Van Duuren et al.* 1972). Now stearic acid gave no sarcomas, but methyl stearate and a-stearolactone induced sarcomas in less than 10 per cent of the animals. This unexpected result could not be explained by a chemical reactivity of the compounds. The esterification of the carboxyl groups can, however, result in great changes in the hydrophilic-lipophilic properties and, consequently, in diffusion and transport in tissues and cells.

Our injection experiments were markedly affected by the appearance of local necrosis which was not disclosed in the course of the preliminary tests. Temporary interruption of the injections became necessary to avoid necrotic ulceration of the overlying skin. In mice receiving the higher total dose (0.185 ml) of fatty acid methyl esters, no tumours appeared at the injection site within the observation period of two years. The only two sarcomas induced by the subcutaneous injections appeared in group I 10 which had received the lower total dose (0.025 ml) of MOO given by 5 weekly injections. One sarcoma contained remnants of the injected material. The survival rate in this group was not very high.

A similar phenomenon has been described by *Swern et al.* (1970) who, in their studies of the carcinogenicity of fatty acids, observed that the largest number of sarcomas appeared in the groups that received the smaller doses of the compounds. *Swern et al.* were unable to interprete these findings. A possible explanation of the inverse relationship with dose observed in our experiment could be that the local necrosis, most severe after application of MOO, may have impeded a cancerogenic process.

The incidence of skin papillomas after application of 50 µg of DMBA was not increased by the subsequent injections of methyl esters. The latency period was, however, lowered in the mice injected with MO and, especially with MOO in the higher total dose. It is also observed that the few malignant skin tumours were seen in animals injected with a fatty acid methyl ester after initiation.

The injection experiment showed no correlation between the incidence of malignant lymphoma and treatment. The latency period preceding the first death among animals with lymphoma was only significantly lowered when the animals were exposed to DMBA. This is in accordance with previous results (*Arffmann & Glavind* 1974). No evidence

for the influence of sex on the results of the injection experiment was found.

The results indicate that MOO has a weak cancerogenic effect on the subcutaneous tissue of mice. The activity seems to be that of promotion conditioned by initiation by a carcinogen applied to the skin and possibly to other sites. The influence of injected fatty acid methyl esters on a distant process of carcinogenesis, e.g., in the skin, seems possible, *though it presumably* must be weak.

The present experiments show that the fatty acid methyl ester previously found to be most active in tumour induction if applied to skin is also the most active compound if tested after application via other routes. The cutaneous administration revealed MOO *as a weak carcinogen and a* potent promoter (*Arffmann & Glavind* 1974). The present results suggest a promoter effect of the compound on the exposed tissues *after* peroral and subcutaneous application. A possible effect on the stomach presents the most important pertinent problem concerning carcinogenic hazards to man and more extensive studies should be conducted, especially in this field.

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A CYTOGENETIC STUDY OF GONADOBLASTOMA TISSUE IN TWO CASES

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Philip, J Hansen, M. K. & Reintoft, I A cytogenetic study of gonadoblastoma tissue in two cases. Acta path. microbiol. scand. Sect. A 83 559-567 1975.

The hypothesis that gonadoblastoma (gonocytoma III) arises from gonadal tissue with a male chromosome complement has earlier been forwarded. In the literature there is no case with a well documented diagnosis of gonadoblastoma and absence of a Y chromosome. In the two presented cases, one a phenotypic female the other a phenotypic male gonadoblastoma was diagnosed. Cytogenetic studies of the removed gonadoblastomas revealed a Y chromosome in both cases. This is in accordance with the hypothesis.

Key words Gonadoblastoma; cytogenetics.

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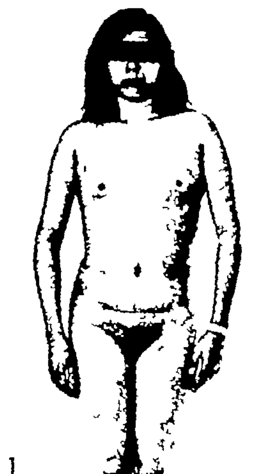
Gonadoblastoma is a tumor of germ cell origin (*Scully* 1953) It is characterized by the presence of germ cells, sex cord cells (granulosa-Sertoli like) and most often Leydig cells. *Teter* has reclassified some germ cell tumors (1960) including the gonadoblastoma which he calls gonocytoma III but *Scully* (1970) rejects this classification.

Based on the literature and a study of five cases *Philip & Teter* (1964) forwarded the hypothesis that gonadoblastoma originated from gonadal tissue with a male chromosome complement (46, XY or complements including a Y chromosome) i.e. gonadoblastoma tumors are supposed to originate from dysgenetic testes The study was based on cells from peripheral tissues, not on cells from the gonadoblastoma. It is understood that the microscopic picture itself does not yield any solution to the origin.

On the basis of the literature and cytogenetic investigation this time of gonadoblastoma tissue from two new cases, the problems of the genetic sex of the gonadoblastoma, still thought to be male, will be reviewed.

Case History 1

Eighteen year old phenotypic female a little virilized admitted because of primary amenorrhoea (Fig. 1) Shoulders were broad and pelvis slender and she had well developed muscles. (Height 168 centimetres, weight 59,2 kilograms) The upper lip was a little hairy the pubic hair had feminine demarcation, but was, however rather strong towards anus and thighs. Clitoris was normal and vagina had normal depth with a small portio the mucous lining of vagina showing estrogen influence. The breasts were small and showed no development, nor estrogen influence There were no hernias. Uterus was felt infantile and to the left a moderately enlarged, mobile, indolent ovary was found. The right ovary could not be felt.





X-ray of the pelvis showed no calcifications, and intravenous pyelografi was normal. Her bone age was estimated to 15 years. Hormone analyses showed elevated excretion of pituitary gonadothrophins in urine 80 and 76 mouse units/24 hours—normal upper limit 60 units/24 hours. The urinary 17-ketosteroids and 17-ketogenic steroids were within the normal range. Serum—testosterone was high, but within the normal range. The urinary excretion of estrogen was 0 and 2 mikrograms per 24 hours.

At laparotomy a small uterus and normal tubes were found. The right ovary was a streak gonad, the left ovary consisted of two intimately connected tumors both with smooth surfaces and mobile. Salpingoophorectomy of the left side and biopsy of the streak on the right side were carried out. Four months later second look laparotomy was done, there was no recurrence of the tumors on the left side and no signs of spread intraperitoneally. The right ovary appeared unchanged and was removed together with the remaining tube and the uterus. The tissue from the right ovary was handled according to the description later in this article under Chromosome Investigation. The postoperative course was uneventful.

Pathology

The uterus and the tubes were macroscopically and microscopically normal.

Ovaries. The left ovary was changed into two round tumors measuring 2 × 2 × 2½ centimetres and 3 × 3 × 4 centimetres. The smaller tumor was lobulated and firm with the cut surface showing solid homogenous grey-white appearance, the larger tumor had many cysts the size of a pea, smooth walled containing clear fluid. Between the cysts there were firmer fibrous, white streaks and unsharply limited, soft, whitish areas.

The right ovary consisted of the biopsy and the streak removed later on and was of solid firm consistency with homogenous yellowish-grey cut surface.

Microscopically both ovaries were built up of tissue with collagen fibres, but within these the following cell groups were arranged germ cells, granulosa-Sertoli cells and Leydig cells (Fig. 2).

The granulosa-Sertoli cells were arranged around one single cell of germ cell appearance, but also around larger groups of such cells and around eosinophilic vacuoles i.e. Call-Exner bodies. Calcifications were found in the cell groups, in some places totally replacing those cells (Fig. 3).

In the smaller solid tumor gradual transition from larger groups of germ cells surrounded by a distinct coherent rim of granulosa-Sertoli cells to tumor tissue, totally built up by germ cells, were seen. Small septa of connective tissue stroma with infiltration of lymphocytes separated the tumor tissue (Fig. 4).

The larger tumor partly of cystic appearance, had squamous epithelium, pseudostratified cylindrical epithelium, and intestinal surface epithelium on the inner sides of the cysts. Other areas did not show characteristic tissue formation. The intervening tissue consisted of connective tissue with larger and smaller areas of cartilage as well as small groups of germ cells and granulosa-Sertoli cells arranged as described above (Fig. 5).

The histological diagnosis is gonadoblastoma with dysgerminoma and teratoma of the left ovary and fibrous streak with gonadoblastoma of the right ovary.

Case History II

Twentyfive year old phenotypic male admitted for bilateral inguinal hernias (Fig. 6).

He had narrow shoulders and broad hips. Puberty occurred at the age of 14. He shaved daily. He had been married one year and complained of sterility. His sexual life was otherwise normal (Height 178 centimetres, weight 78 kilograms). Penis was normal, the pubic hair not abundant and horizontally demarcated. An ejaculate showed aspermia. Scrotum was empty but in the vertical position a small intumescens was felt on the right side. Hormone analysis showed low excretion of pituitary gonadothropins, less than 8 mouse units/24 hours. The urinary 17-ketosteroids, 17-ketogenic steroids as well as estrogenic substances were normal.

At herniotomia an open processus vaginalis and, partly inside the abdomen, a small gonad, macroscopically an ovary with salpinx and ovarian ligaments, was seen, and a laparotomy performed. On the left side similar structures and in the midline a rudimentary uterus were found. Both gonads had small granular intumescentias on the surface.

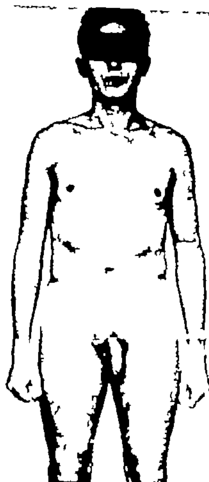
Fig. 1. Patient No 1 (ADL – 280154).

Fig. 2. Group of Leydig Cells in Connective Tissue in Upper Part. In Addition at the Bottom a Group of Germ Cells and Some Granulosa Cells × 260 Hem. Eos.

Fig. 3. Right Ovarium: Fibrous Streak with Gonadoblastoma Containing Call-Exner Bodies × 105 Hem – Eos.

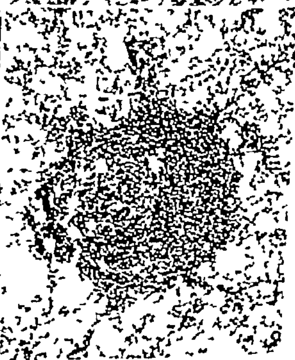
Fig. 4. Dysgerminoma of Typical Appearance × 105 Hem. Eos.

Fig. 5. Teratoma with Cyst with Different Kinds of Epithelial Lining Cells and Often Containing Horny Material. In the Center an Island of Cartilage. Close to the Teratoma Formation Groups of Gonadoblastoma Cells May Dimly Be Seen at the Bottom × 40 Hem. Eos.


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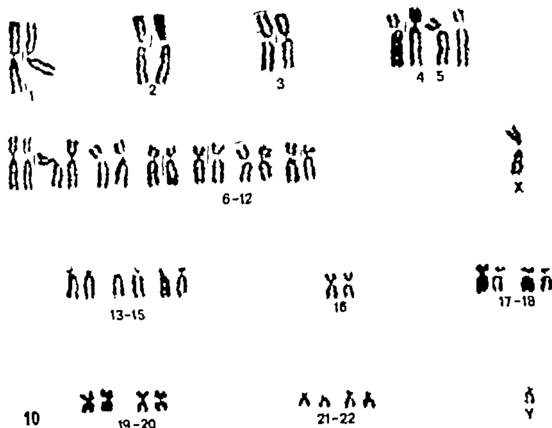


Fig 10 Karyotype of Patient No I

Biopsies from both gonads were taken, and, as the microscopic findings showed gonadoblastoma, second operation was performed and the internal genitalia were removed. The gonads were removed as described under Chromosome Investigation. The pituitary gonadothrophins rose immediately but with androgen substitution he has no sexual problems.

Fig 6 Patient No II (HJ – 190242)

Fig 7 From the Abdominal Gonad. To the Left Some Testicular Tubules and Groups of Leydig Cells and to the Right Gonadoblastoma Tissue Are Seen. x 90 Hem – Eos

Fig 8 Group Consisting of Few Germ Cells, and of Granulosa-Sertoli Cells, the Last Ones Also Surrounding a Call-Exner Body x 500 Hem – Eos.

Fig 9 Seminoma of Typical Appearance x 90 Hem Eos.

Pathology

The uterus was rudimentary with normal cylindrical epithelium in the cervix. From its middle running to the gonads on both sides histological typical ductus deferentes were found. The lef one was laterally transformed to epydydimus and in the uterine cervix both passed to seminal vesicles. From the left uterine corner a uterine tube set off.

Gonads: Biopsies from the gonads showed a fine fibrous stroma containing tumor tissue somewhat lobular as the tumors were divided by connective tissue into smaller areas. The tumor tissue was built up as the gonadoblastoma tissue in case I (Fig. 7 & 8) Tubular structures, sometimes very close to normal testicular tubules without spermatogenesis, were seen as well in the middle as in the periphery of the tumor On both sides some areas of the gonads were dominated by seminoma-dysgerminoma tissue diffusely infiltrated with lymphocytes and with some granulomas (Fig. 9)

The histological diagnosis of the gonads is gonadoblastoma with seminoma.

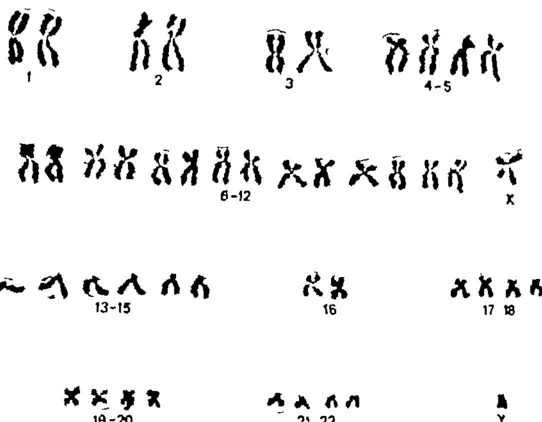


Fig 11 Karyotype of Patient No 11. Both Karyotypes Were Done Before Modern Chromosome Staining Techniques Became Available. Figs. 10 & 11 Both Demonstrate That the Chromosome Complement of Both Patients Were 46 XY It Should Be Noted That They Are Both From Cells Obtained From Tissue Cultures From Gonadal Tumor Tissue.

TABLE 1 *Chromosome Count Distribution in Cells from Case 1 Blood and Gonadal Tissue*

Tissue	No of Cells with 42	43	44	45	46	Total	No of Cells analysed
Blood		1		1	18	20	4
Gonadal Tissue							
Right gonad							
Biopsy 1	1			2	17	20	2
Biopsy 2					4	4	1
Biopsy 3 (+ gonadoblastoma*)	1				19	20	2
Biopsy 4 (+ gonadoblastoma)			2	2	16	20	2
Biopsy 5							unsuccessful
Biopsy 6			1	3	8	12	2
Total	2	1	3	8	82	96	13

* by histological examination.

TABLE 2. *Chromosome Count Distribution in Cells from Case II Blood Skin and Gonadal Tissue*

Tissue	44	45	46	47	Total	No of Cells analysed
	No of Cells with					
Blood		2	23		25	3
Skin		2	23		25	3
Right Gonad						
Biopsy 1			10		10	3
Biopsy 2		1	9		10	5
Left Gonad						
Biopsy 1			10		10	3
Biopsy 2	1		9		10	3
Biopsy 3		1	9		10	3
(+ gonadoblastoma)						
Biopsy 4		1	9		10	3
Biopsy 5		2	46	2	50	6
Total	1	9	148	2	160	32

by histological examination.

Chromosome Investigation

The karyotypes of the patients from case I and II respectively are seen in Figs. 10 and 11

Results of chromosome counts are given in Tables 1 and 2. Both cases were operated on twice with a view to remove the remainder of the gonads after the diagnosis was made. Tissue from the gonads could therefore be obtained and was carefully cut into several pieces. Half of each piece was cultured for chromosome analysis, and half was histologically examined. By this method it was ensured that tumor tissue was actually investigated. In both cases successful cultures were derived from gonadoblastoma tissue. The modal number was 46 and the karyotype 46, XY with normal autosomes in all tissues examined.

Both patients had a buccal smear examined for Y-chromatin. 200 cells were counted for each patient with no Y-chromatin positive cell found. The Y-chromatin investigation was not available at that time and the slides do not exist any longer.

DISCUSSION

When the hypothesis of the male origin of gonadoblastoma was first *forwarded*, only a limited number of cases with a complete chromosome analysis were published. Since then a number of publications fulfilling this criterium have appeared, and Scully summarized those published until 1969 (*Scully* 1970) The majority of the published cases have had a Y chromosome. Scully mentions 4 cases in whom no Y was demonstrated, and we have been able to find one more. Table 3 contains the information about these cases.

From the table it is evident that no case with a really well documented diagnosis and careful examination with absence of a Y chromosome has been published until now

Furthermore it must always be borne in mind that mosaicism may be present without being demonstrated, because the crucial tissues were not investigated. The two cases included in this paper are of interest in this discussion because they are the first in which the tumor itself was investigated cytogenetically

In our opinion the hypothesis still holds true that gonadoblastomas (gonocytoma III) originate from gonads that are dysgenetic testes. We are not claiming that gonadoblastomas cannot arise in individuals with a 46, XX complement, but the possibility of a not demonstrated Y chromosome must always be borne in mind.

TABLE 3 *Cases Where No Y Chromosome Has Been Demonstrated Referred in the Literature as Exceptions*

Ref.	Microscopy	X-chromatin	Karyotype	Conclusion
Dominguez & Greenblatt 1962	Dysgerminoma Gonadoblastoma?	neg.		No diagnosis of gonadoblastoma
Müller 1964	Gonadoblastoma? No pathological description		XO/X + small metacentric chromosome	Gonadoblastoma diagnosis doubtfull, possibly Y chromosomic material present
McDonough et al. 1967	Gonadoblastoma		45 XO/45 X + fragment	Possibly Y chromosomic material present
Bacalao & Dominguez 1969	Gonadoblastoma		46, XX	No details about the cytogenetic investigation
Patel & Prentice 1972	Dysgerminoma Gonadoblastoma	Only 14 cells counted Karyotype?	No fluorescent study 45,XO/46, XX	Doubtfull cytogenetic diagnosis

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Acta path. microbiol. scand. Sect. A, 83 568–572, 1975

STUDIES ELUCIDATING THE IMPORTANCE OF THYMUS ON THE DEGREE OF INCREASED BLOOD PRESSURE AND VASCULAR DISEASE IN RENAL HYPERTENSIVE MICE

A Comparison of the Disease in Nude and Haired Littermates

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Svendsen, U G Studies elucidating the importance of thymus on the degree of increased blood pressure and vascular disease in renal hypertensive mice. A comparison of the disease in nude and haired littermates. Acta path. microbiol. scand. Sect. A, 83 568–572 1975

The degree of round cell infiltration around hypertensively damaged heart arteries in one kidney Goldblatt hypertensive mice is more pronounced in haired mice with normal thymus function than in their nude littermates with genetic aplasia of the thymus. The level of hypertension and the prognoses for the hypertensive mice are, however not influenced by the presence of thymus and thymus derived T cells. The results give evidence that delayed type immune reactions are involved in the hypertensive vascular disease in mice but fail to support the assumption that they have pathogenic importance for either the level of hypertension or the prognoses of the one kidney Goldblatt hypertensive mice

Key words Renal hypertensive mice thymus blood pressure vascular disease

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A round cell infiltration in adventitia of teries damaged by high intravascular presre has been observed in experimental ani ls as well as in humans (for literature see and 10) It has been proposed that such cell infiltration is caused by immune reactions against substances in the damaged vascular walls (2, 3 4 6 7 8, 9 15 and 16) Experiments with transfer of thoracic duct cells from acute angiotensin II hypertensive rats (6) and renal hypertensive rats (15) give evidence that delayed type immune reactions are involved. Based on these findings it has been proposed that the immune reactions have direct pathogenic importance for the hypertension (5 7)

The purpose of the present investigation has been to elucidate if the degree of hypertension and vascular disease and the prognoses for the hypertensive animals are changed in athymic animals or if the immune reactions merely are auto-immune reactions formed following tissue damage (1 14) without pathogenic importance. For this purpose nude mice are suitable as they lack

recognizable thymus tissue and seem to be depleted (or nearly depleted (11)) of thymus derived T lymphocytes by both functional and histological studies (for literature see 12 and 13) Such nude mice and their haired litter mates were made renal hypertensive by means of a Goldblatt silver clip and contralateral nephrectomy The level of hypertension, the prognoses and the round cell infiltration around damaged arteries in nude and haired mice were thereafter compared.

MATERIAL AND METHODS

Animals. Mice (NMRI SPF GL Bomholtgård Ltd., Denmark) of both sexes, 20–25 gram, carrying the mutant allele (nu/nu) and their haired littermates (nu/+) received tetracyclin in the drinking water (100 mg/l)

The animals were divided into groups as follows

Group I *10 nude and 10 haired untouched control mice*

Group II *31 haired female mice unilaterally nephrectomized with 0 10 mm Goldblatt clips on their remaining renal artery*

Group III *30 nude female mice unilateral nephrectomized with 0.10 mm Goldblatt clips on their remaining renal artery*

A *Goldblatt silver clip* with an internal diameter of 0.10 mm was applied on the left renal artery of the mice in groups II and III in penthymal anaesthesia The kidney was placed subcutaneously 4 days later contralateral nephrectomy was performed. The further treatment was the same for all mice in the 3 groups. Body weight was recorded each week. Before sacrifice *blood pressure* was recorded. In light ether anaesthesia a catheter was placed in the left carotid artery and connected to a Tybjerg Hansen capacitance pressure transducer (Simonsen & Weel, Copenhagen) and a O-14 graphic recorder (Dansbridge, Copenhagen) Blood pressure was recorded for one hour in the conscious unrestrained animal. Most animals were investigated around day 10 to 15 (Fig. 2) but at signs of sickness (thin, pathetic, hunchbacked, forced respiration) blood pressure was measured immediately After sacrifice the relative heart weight (heart weight × 100/body weight) was determined and the heart, kidney and pancreas were fixed in 4 per cent formalin and embedded in paraffin. Five micron thick sections were cut and stained with Van Gieson Hansen (VGH) and the periodic acid Schiff (PAS) stain. The round cell infiltrations around arteries in the heart were graded semiquantitatively according to a photographed scale from 1+ to 3+ in which a 1+ cellular reaction includes the sparse mononuclear reaction in adventitia of normal arteries (Fig. 1a, b and c) For comparison of experimental results the student's t-test was used. The 5 per cent level was used as indicative for significant differences.

RESULTS

Mice dying during the operations or in the first 48 hours after contralateral nephrectomy were not included in the material. The *mortality* in the first 48 hours was equal in the nude and haired mice, and increased with increasing body weight. The *state of health* for the mice was equally bad. 9 nude and 11 haired mice died spontaneously and 7 of the nude and 8 of the haired mice in which blood pressure was measured were clinically sick at the time when their blood pressure was recorded. Fig. 2 shows at which time after contralateral nephrectomy the mice in groups II and III died spontaneously or were sacrificed after their *blood pressures* had been measured. It is seen that there is no significant difference between the levels of blood pressure in the nude and haired mice throughout the observation time. The mean blood pressure was significantly and similarly increased in renal hypertensive nude (156 ± 19 mm Hg (SD) (range 120–190 mm Hg) $p<0\ 001$) and haired (159 ± 26 (SD) mm Hg (range 90–200 mm Hg) $p<0.001$) mice, as compared with the respective control group of nude (112 ± 17 (SD) mm Hg (range 90–140 mm Hg)) and haired (111 ± 14 (SD) mm Hg (range 90–140 mm Hg)) mice. The *mean relative heart weight* was significantly higher in nude (0.46 ± 0.05 (SD) per cent, (range 0.40–0.54 per cent) $p<0.01$) than in haired (0.39 ± 0.06 (SD) per cent (range 0.33–0.52 per cent)) control mice. The mean relative heart weights were significantly increased in both the renal hypertensive nude ($0.59 \pm 0\ 11$ (SD) per cent, (range 0 47–0.98 per cent) $p<0.001$) and haired ($0.57 \pm 0\ 09$ (SD) per cent (range 0.37–0 77 per cent) $p<0.001$) mice as compared with the respective control group The

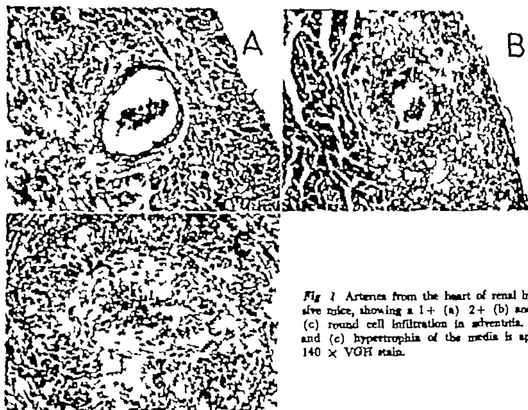


Fig 1 Arteries from the heart of renal hypertensive mice, showing a 1+ (a) 2+ (b) and a 3+ (c) round cell infiltration in adventitia. In (b) and (c) hypertrophia of the media is apparent. 140 × VOH stain.

heart weight was increased due to hypertrophia of the heart muscle.

Microscopic investigation Group I (control mice) The heart kidney and pancreas no vascular disease. *Group II (renal hypertensive haired mice) The heart* a 3+ cellular reaction was observed in 13 of 31 (42 per cent) mice (Fig. 2) A 2+ cellular reaction was found in 4 mice (13 per cent) The cells in the infiltrates had a morphology like great lymphocytes or monocytes, but few fibroblasts were observed too (Fig. 1 b and c) The 3+ cellular reactions were observed between day 5 and 16 (Fig 2) Heart infarcts were found in 21 mice (57 per cent) of which 9 were found dead and 7 of the remaining 12 were clinically sick at the moment of blood pressure recordings. Fibrinoid degeneration of the media, increasing to total necroses, was found in 22 (71 per cent) of the mice. Hypertrophia of the arterial wall with increased ratio wall/lumen was often observed (as seen by comparison of Fig. 1 a and c) *The kidney* degenerative changes consisting of hyaline casts, thickened and wrinkled basement membranes of the tubules, increased amounts of PAS positive material in the glomerular tufts and small infarcts were found in some animals. *The pancreas* in 2 mice the number of round cells around arteries were found to be increased. *Group III (renal hypertensive nude mice) The heart* a 3+ cellular reaction was observed in only 1 (3 per cent) mouse, which had blood pressure of 145 mm Hg when investigated on day 10 after contralateral nephrectomy and which clinically looked healthy (Fig 2) A 2+ cellular reaction was observed in 4 mice (13 per cent) The morphology of the cells was similar to that observed in the haired mice. Heart infarcts were found in 17 mice (68 per cent) of which 7 were found dead and 4 of the remaining 10 mice were clinically sick at the moment of the blood pressure recordings. Fibrinoid degeneration of the media, increasing to necroses, were found in 15 (50 per

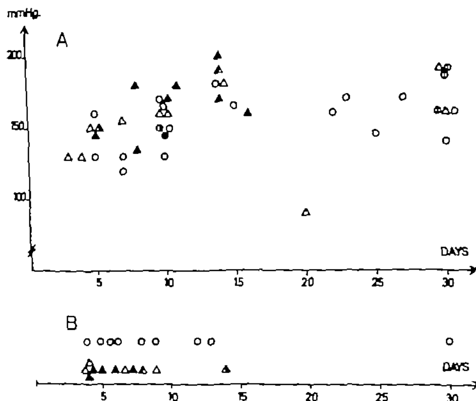


Fig. 2 The time in days after contralateral nephrectomy at which the blood pressure was measured (A). It is seen that the 3+ cellular reactions were observed early in the observation time. 11 haired and 9 nude mice died spontaneously and nearly all of them had heart infarcts (B). The nu/nu mice are marked with circles, the nu/+ with triangles. The symbols further show if the cellular reaction was 1+ (○ and △) 2+ (⦶ and ⟁) or 3+ (● and ▲) reaction.

cent) of the mice. Hypertrophia of the arterial wall with increased ratio wall/lumen was often observed. *The kidney*: degenerative changes were observed as in the haired mice. *The pancreas*: an increased number of round cells around arteries was found in 2 mice.

DISCUSSION

In the present investigation one kidney renal hypertension was induced by help of silver clips with internal diameters of 0.10 mm. The reason for using 0.10 mm clips was that broader clips only seldomly produced pronounced vascular disease and that the mice did not survive application of narrower clips. Adult female mice and a short observation time eliminated the effect of increasing body weight due to age: no difference in either weight gain or weight loss between the nude and haired renal hypertensive mice was observed. The 0.10 mm clips induced a very malignant type of hypertension in both groups (great numbers of heart infarcts and spontaneous deaths) and a marked vascular disease developed rapidly. Furthermore, the mortality after contralateral nephrectomy, the level of hypertension and the malignity of the disease were found to be quite similar in the nude and the haired mice. A marked difference was, however, found in the degree of round cell infiltrations around damaged heart arteries. While a 3+ degree of reaction was only observed in 1 nude mouse (5 per cent), 15 (42 per cent) of the haired mice showed this reaction. Contrary to the difference in

the degree of round cell infiltrations between nude and haired mice, the two groups showed similar degrees of fibrinoid degeneration of media. The present results support the assumption that delayed type immune reactions play a role for the degree of round cell infiltration around hypertensively damaged arteries in one-kidney Goldblatt hypertensive mice however this reaction does not seem to have any pathogenic importance either for the level of hypertension or for the prognoses, as estimated by the number of heart infarcts and the mortality The findings do however not exclude that the delayed type immune reactions might be of pathogenic importance under conditions of less malignant hypertension and a more prolonged observation time.

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SERUM ALPHA-FOETOPROTEIN AS A MARKER FOR ENDODERMAL SINUS TUMOUR (YOLK SAC TUMOUR) OR A VITELLINE COMPONENT OF 'TERATOCARCINOMA'

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Nørgaard-Pedersen, B., Albrechtsen, R. & Teilum, G. Serum alpha-foetoprotein as a marker for endodermal sinus tumour (yolk sac tumour) or a vitelline component of 'teratocarcinoma'. Acta path. microbiol. scand. Sect. A, 83 573–589 1975

The correlation between serum alpha-foetoprotein (AFP) and the clinical pathological findings of 24 germ cell tumours arising from the testes (14 cases), the ovaries (5 cases), the mediastinum (3 cases), the retroperitoneal region (2 cases) and the sacrococcygeal region (2 cases) are presented. Irrespective of marked differences in age and sex of the patients, primary site of the tumours and clinical outcome, the 24 cases constituted a homogeneous group in fundamental histological patterns and in AFP synthesis. In all cases of endodermal sinus tumour or teratocarcinomas with a distinct vitelline component an increased serum AFP concentration was found in the pre-operative serum samples. AFP was also demonstrated in the tumour tissue by quantitative determination of AFP in tumour homogenate (5 cases) and, by immunofluorescence technique, positive staining of the cells lining the endodermal sinuses and of the hyaline globules was found (5 cases). In 12 germ cell tumours without vitelline components in the tumour tissue sections, a normal AFP concentration below 20 μg/l was found in pre-operative serum samples.

Key words: Alpha-foetoprotein; endodermal sinus tumour; teratocarcinoma.

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The association of alpha-foetoprotein (AFP) with 'teratocarcinomas' of ovary and testis was simultaneously and independently demonstrated by *Abelev et al.* (1967) and *Masopust et al.* (1968). By now it is well-established that AFP is a biological marker of cells responsible for its synthesis in early embryogenesis as well as for certain gonadal and extragonadal germ cell tumours. Of decisive importance for the development of a rational basis for the clinical application and further elucidation of the problem is 1) the cellular source of AFP in ontogenesis, 2) the cellular source in malignancy depending on an exact tumour histogenesis and 3) the embryological basis for the neosynthesis of AFP in germ cell tumours.

The first point has been clarified in the works by *Gitlin & Boesman* (1967) and *Gitlin*

& *Parricelli* (1970) who demonstrated that the synthesis takes place in the human yolk sac.

As to the other two points, the attention has been focused on Teilums concept of endodermal sinus tumour (1959 1965 1971) as a germ cell tumour showing a specific differentiation of yolk sac endoderm which would produce AFP analogous with the phy siological AFP synthesis by the foetal yolk sac in early embryogenesis (25 26) Several reports have confirmed that patients with gonadal endodermal sinus tumours always show elevated AFP concentration in serum (*Ballas* 1972, *Wilkinson et al* 1973 *Ballas* 1974 *Itoh et al.* 1974 *Teilum et al.* 1974 *Talerman & Haije* 1974) An increased serum AFP concentration has also been found in patients with extragonadal endodermal sinus tumours (*Tsuchida et al.* 1973 *Itoh et al.* 1974 *Talerman & Haije* 1974 *Scully & McNeely* 1974)

Furthermore, immunofluorescence studies of pure and typical endodermal sinus tumours in human subjects have provided evidence that AFP is mainly produced by the cells of the yolk sac endoderm lining the endodermal sinuses. (*Itoh et al.* 1974 *Teilum et al.* 1974)

In view of these recent studies it appears that determination of AFP in serum from patients with germ cell tumours can be used as a marker for the presence of a vitelline component in these tumours. In order to investigate this connection further it was decided to correlate AFP quantitation in sera and tumour homogenate with an exact histological classification of these tumours in gonadal as well as in extragonadal sites. Furthermore, the cellular basis for the AFP production has been studied by means of immunofluorescence technique in some of the AFP producing tumours.

MATERIAL

Serum and tumour tissue specimens from patients with gonadal as well as extragonadal germ cell tumours were obtained from various surgical departments. Serum samples were taken before operation and during the postoperative period from patients subjected to or not subjected to chemotherapy. Several patients have been followed by serum AFP estimation for more than 6 months. Immunofluorescence studies were carried out only on tumour tissue collected at operation.

METHODS

Quantitation of AFP in Serum and Tissue Extracts

AFP determinations were carried out by a radio-immunoelectrophoretic (RIEP) technique recently described by *Nørgaard-Pedersen* (1974) The sensitivity of the assay is 20 μg/l. In sera with AFP levels above 1000 μg/l, quantitations were carried out by rocket immunoelectrophoresis (*Nørgaard-Pedersen* 1972) Comparable results were obtained by the two methods. The same standards and antibodies were used in both techniques.

Antigen α_1-foetoprotein standard 0.5 and 220 mg/l (Behringwerke AG Marburg) was used as primary standard.

Antibody Rabbit immunoglobulins to human α_1-foetoprotein (Dakopatts, Copenhagen)

Immunofluorescence (IF) Technique for Localization of AFP

The IF staining was carried out as a double layer technique as described elsewhere (25)

First layer Rabbit immunoglobulins to human AFP (Dakopatts, Copenhagen)

Second layer Fluorescein conjugated swine-against-rabbit serum IgG (Dakopatts, Copenhagen)

Cryostat cut tissue sections fixed in 4 C 70 per cent ethanol for a few minutes as well as paraffin embedded tissue sections prepared according to *Sainte-Marie* were used

Classification of Yolk Sac Tumours

Based on comparative studies of human ovarian and testicular tumours, *Teilum* (1959) introduced the concept of yolk sac tumours (endodermal sinus tumours) into the pathology of germ cell tumours as a specific subgroup of embryonal carcinoma. Since the totipotential embryonal cells of embryonal carcinoma are capable of differentiation into teratoma or choriocarcinoma in a way analogous to the morphogenesis of the embryo and its (extra-embryonic) trophoblast (23) it seemed logical that these cells should also be able to differentiate into other types of tumours of the extra-embryonic foetal membranes such as yolk sac tumours and mesoblastomas.

Teilum (1965 1971 classified the germ cell tumours of the testis as seminoma, embryonal carcinoma, endodermal sinus tumour choriocarcinoma and teratoma, while the poorly differentiated ca-

TABLE 1 *Histological Features of Gonadal Germ Cell Tumours with Increased Serum AFP Concentration*

Case no.	Sex	Age month (mo) year (yr)	Localization	Histological diagnosis	Alpha-foetoprotein µg/l
1	♂	21 mo	Infant testis	Endodermal sinus tumour	2210
2	♂	9 mo	Infant testis	Endodermal sinus tumour	3800
3	♂	10 mo	Infant testis	Endodermal sinus tumour	2000
4	♂	36 yr	Adult testis	Teratocarcinoma with endodermal microcysts and embryoid bodies	1375
5	♂	31 yr	Adult testis	Embryonal carcinoma with endodermal sinus structures, labyrinthine pattern and vacuolated network	7950
6	♂	21 yr	Adult testis	Embryonal carcinoma (lung metastasis) with marked vitelline components of endodermal sinus structures	560
7	♂	27 yr	Adult testis	Embryonal carcinoma with foci of vitelline components and embryoid bodies	370
8	♂	32 yr	Adult testis	Embryonal carcinoma with areas of endodermal sinus structures and vacuolated network	5630
9	♂	23 yr	Adult testis	Embryonal carcinoma with a few areas of vitelline components (vacuolated network, endodermal sinus structures)	400
10	♂	29 yr	Adult testis	Embryonal carcinoma with teratoma and seminoma. A few areas of vitelline components (EST structures and vacuolated network)	21750
11	♂	27 yr	Adult testis	Embryonal carcinoma with a few vitelline components (EST structures)	6200
12	♂	28 yr	Adult testis	Embryonal carcinoma with a few areas of vitelline components (EST structures)	1880
13	♂	24 yr	Adult testis	Teratocarcinoma with vitelline components (vacuolated network)	1700
14	♂	38 yr	Adult testis	Embryonal carcinoma with a few vitelline components	510
15	♀	20 yr	Ovary	Tumour of combined type (EST associated with choriocarcinoma and dysgerminoma)	950
16	♀	17 yr	Ovary	Endodermal sinus tumour combined with dysgerminoma	7100
17	♀	14 yr	Ovary	Endodermal sinus tumour combined with embryonal carcinoma and dysgerminoma	2360

*EST Endodermal sinus tumour

bryonal carcinoma was considered developmental stage of the three latter types. Any combination of the various histological types of germ cell tumour may occur. The often used term 'teratocarcinoma' indicates an embryonal carcinoma combined with teratoma. It is also believed that a histogenic relationship between seminoma and the other types of germ cell tumour exists.

After it had been recognized that endodermal sinus tumours in the ovary and the adult testis

37*

TABLE 2. *Histological Features of Extragonadal Germ Cell Tumours with Increased Serum AFP Concentration*

Case no.	Sex	Age month (mo) year (yr)	Localisation	Histological diagnosis	Alp fetop µg
18	♂	32 yr	Anterior mediastinum	Embryonal carcinoma with vitelline components (vacuolated network)	40
19	♂	21 yr	Anterior mediastinum	Teratocarcinoma and germinoma with several areas of vitelline components	39
20	♂	28 yr	Anterior mediastinum	Severe necrotic embryonal carcinoma	57
21	♂	27 yr	Retroperitoneum	Polycystic teratoma with foci of endodermal cysts and endodermal sinus structure	46
22	♂	23 yr	Retroperitoneum	Teratocarcinoma with a large vitelline component (endodermal sinus structure vacuolated network and labyrinthine structure)	160
23	♀	12 mo	Sacrococcygeal region	Endodermal sinus tumour	169 00
24	♀	19 mo	Sacrococcygeal region	Endodermal sinus tumour	700

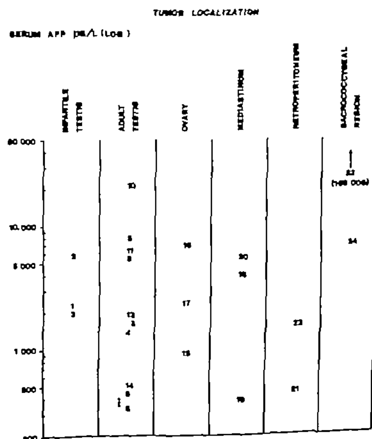


Fig. 1. Serum AFP concentration in germ cell tumours of different localization all having vitelline components of various patterns. The numerals refer to the numbers of the patients (see case stories)

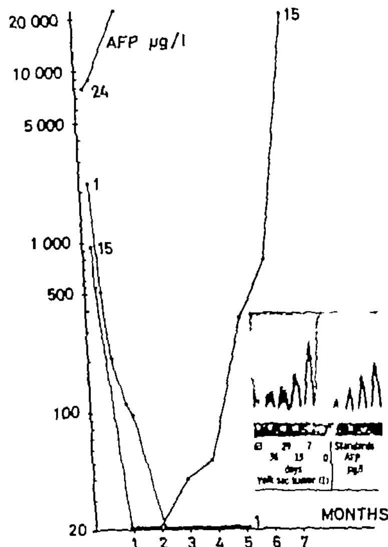


Fig 2 Serum AFP concentrations in cases 1 15 and 24 before and after operation. Inset an autoradiogram of the radioimmunoelectrophoretic quantitation of AFP in the serum samples from case 1 before and 7 to 63 days after operation. AFP standard 40–320 µg/L

were identical It has been realized that the so-called embryonal carcinoma of the infantile testis (or orchioblastoma) is also a yolk sac tumour mimicking the endodermal sinus structures in the adult (*Huntington et al* 1963) Furthermore, endodermal sinus tumours have been observed in number of extragonadal sites, including the anterior mediastinum, the sacrococcygeal region, the retroperitoneum, the infant vagina, the pineal gland and the stomach. In all these locations, the histological interpretation has been based primarily on the presence of the endodermal sinus tumour pattern which in immunofluorescent studies now has been found to be associated with the synthesis of AFP (25 27)

RESULTS

The serum AFP concentration was found to be increased in 24 patients with germ cell tumours of different localization (Fig 1 and table 1 and 2) Histological examination of all these tumours showed distinct endodermal sinus patterns except in case 20 where the tumour was very necrotic. The clinical findings, the histological examinations and the laboratory values are given below in the case reports and in tables 1 and 2

In most cases, serum AFP was determined before operation and several times after surgery see Fig. 2. In some cases, the AFP



Fig 3 Endodermal sinus tumours of the infant testis showing: A. Typical endodermal sinus structures. Serum AFP 2210 μg/l. Case 1 (21-month-old boy) H.E., × 84 B. Vacuolated network of honeycomb appearance. Case 1 H.E × 10. C. Endodermal sinus structure. Serum AFP 2000 μg/l. Case 3 (10-month-old boy) H.E., × 210.

concentration was also determined in tumour homogenate (case 1 3 9 14 24) Immunofluorescence studies were carried out in cases 1 9 and 24

Case Reports of 24 Germ Cell Tumours with Elevated Serum AFP Concentrations

Gonadal Tumours

1 *Infant Testis*

Case 1 A 21-month-old boy was admitted to the hospital on an initial diagnosis of hydrocele testis developed within the last 8 days. At operation, a tumour measuring 3 × 3 × 2 cm was found in the right testis. The cut-surface of the tumour was marbled, dark-grey and of a soft consistency

Histological examination Endodermal sinus tumour of the infant testis (Fig 3 A and B) The serum AFP concentration was 2210 μg/l before operation and less than 20 μg/l 10 months after operation (Fig.)

This case has previously been described (23) By immunofluorescence microscopy a bright green staining of the cells lining the endodermal tissues and of the hyaline globules was found. The AFP concentration in tumour homogenate was 3.8 μg per g wet weight of tumour tissue

Case 2 A 9-month-old boy was admitted to hospital because hydrocel testis was suspected. During the last month, a swelling had developed in the left side of the scrotum At operation, a tumour was removed from the left scrotum.

Histological examination Endodermal sinus tumour of the infant testis.

The AFP concentration was 5800 μg/l before operation and less than 20 μg/l 8 months after operation

Case 3 A 10-month-old boy in whom a left scrotal enlargement had developed within the last two months At operation, a tumour was removed. Chest roentgenogram normal

Histological examination Endodermal sinus tumour of the infant testis (Fig 3 C)

The AFP concentration in preoperative serum was 2000 μg/l and two months after operation the AFP concentration was less than 20 μg/l.

The AFP concentration in tumour homogenate was 1.0 μg per g wet weight of tumour tissue

2. Adult Testis

Case 4 A 36-year-old man who had observed a gradual enlargement of the left testis during the last six months. At the age of 12–13 years, the patient had been treated with hormones for retention of the testes. At operation, a 4 × 3 × 3 cm firm lobulated tumour was found in the central part of the testis and invading the epididymis. Post operatively lymphangiography gave rise to a suspicion of metastases to the preaortic abdominal lymph glands.

Histological examination Teratocarcinoma with endodermal microcysts and embryoid bodies.

Serum AFP concentration 1975 µg/l.

Case 5 A 31-year-old man who had noticed enlargement of the left testis during the last two months.

A left orchiectomy was performed. The tumour was necrotic and haemorrhagic and the greater part of the testis and epididymis was replaced by tumour tissue.

Histological examination Embryonal carcinoma with endodermal sinus structures, labyrinthine and honeycomb pattern (Fig. 4 C and D) Serum AFP concentration 7950 µg/l.

Case 6 A 21-year-old man who 20 months earlier had been subjected to operation for a tumour in the left testis (embryonal carcinoma) was admitted to hospital because of acute respiratory failure. Chest roentgenogram revealed metastases to the mediastinum. The patient died a few days after admission. At autopsy multiple metastases to the lungs were found

Autopsy microscopy of lung metastases Embryonal carcinoma with marked vitelline components of endodermal sinus structures.

At the second admission to the hospital the serum AFP concentration was 360 µg/l.

Case 7 A 27-year-old man who two weeks previously had observed an enlargement of the left testis At operation, the entire left testis was replaced by a necrotic and haemorrhagic tumour

Histological examination. Embryonal carcinoma with foci of vitelline components and embryoid bodies

Serum AFP concentration 2 days after operation 370 µg/l Three months after operation, the serum AFP concentration was found to be less than 20 µg/l

Case 8 A 32-year-old man in whom enlargement of the right testis had been noticed two month earlier A right orchiectomy was performed. The necrotic tumour measured 5 cm in diameter

Histological examination Embryonal carcinoma with areas of endodermal sinus structures and vacuolated network. (Fig 4 A and B)

Serum AFP concentration before operation 3630 µg/l

Case 9 A 23-year-old man who during the last year had noticed a gradual enlargement of the right testis. A right orchiectomy was performed; the size of the testis was 6 × 5 × 5 cm.

Histological examination: Embryonal carcinoma with a few endodermal sinus structures, honeycomb pattern, and hyaline globules.

Serum AFP concentration before operation 400 µg/l. By immunofluorescence microscopy a positive staining reaction of the cell lining the endodermal sinuses and of the hyaline globules was seen. Areas with solid embryonal carcinoma showed a negative staining reaction (Fig. 9)

The AFP concentration in tumour tissue homogenate was 0.7 µg per g tissue.

Case 10 A 29-year-old man who was operated upon for a teratoma of the left testis in 1972.

Histological examination of the left testis tumour: Embryonal carcinoma with a few areas of vitelline components (Endodermal sinus structures and honeycomb network) combined with teratoma and seminoma.

February 1974 a metastasis to the left lung was found.

Serum AFP concentration: 21750 µg/l. Radiation therapy was carried out.

Case 11 A 27-year-old in whom swelling of the scrotum had been noticed 3 months previously A left orchiectomy was performed. X-ray showed a metastasis to the left lung

Histological examination Embryonal carcinoma with a few vitelline components (endodermal sinus structures)

Serum AFP concentration 6200 µg/l.

Case 12 A 28-year-old man who had noticed a swelling of the right testis during the last 2–3 months. A right orchiectomy was performed.

Histological examination Embryonal carcinoma with a few areas of vitelline components (endodermal sinus structures)

Serum AFP concentration 1880 µg/l.

Case 13 A 24-year-old man who had noticed a swelling of the left testis. A left orchiectomy was performed and a necrotic cystic tumour was found.

Histological examination Teratocarcinoma with vitelline components (vacuolated network)

Serum AFP concentration: 1700 µg/l.

Case 14 A 38-year-old man who had observed a rapid enlargement of the left testis. Orchiectomy was performed and a necrotic tumour was found.

Histological examination Embryonal carcinoma with a few vitelline components.

Serum AFP concentration before operation 510 µg/l. The AFP concentration in tumour homogenate was found to be 0.6 µg per g tissue.

3. Ovaries

Case 15 A 20-year-old woman in whom abdominal pain had increased during the last 3 weeks.



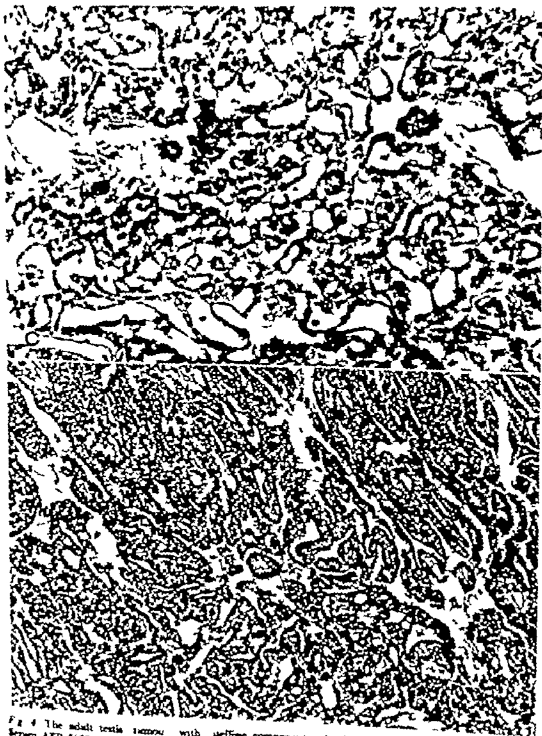


Fig. 4 The adult testis tumour with yolk-sac components. A. Typical endodermal sinus structure. Serum AFP 5630 µg/l. Case 8 (3 -year-old man) H.E., × 525 B. Areas showing typical honeycomb pattern. Case 8. H.E. × 84 C. Labyrinthine and vacuolated structure. Serum AFP 7950 µg/l. Case 3 (31-yea -old man) H.E. × 210 D. Metastases to the lung showing a compressed network of endodermal sinuses Serum AFP 360 µg/l. Case 3 (1-year-old man) H.E., × 84

At operation, a right ovarian tumour (size 18 × 12 × 10 cm) and a left ovarian tumour (size 10 × 7 × 4 cm) were removed. Histological examination. Tumour of combined type (endodermal sinus tumour associated with choriocarcinoma and dysgerminoma) (Fig 7)

Serum AFP concentration a few days after operation 950 µg/l (see Fig. 2)

Urine AFP concentration: 50 µg/l.

Urine HCG concentration 87000 IU/l.

Case 16 A 17-year-old girl in whom slide test on urine for pregnancy was positive was operated upon for an inoperable left side solid ovarian tumour with a few cystic areas measuring about 20 × 15 × 15 cm.

Histological examination Dysgerminoma with areas of typical endodermal sinus tumour

Serum AFP concentration 14 days after operation 7100 µg/l.

Urine AFP concentration 70 µg/l.

Case 17 A 14-year-old girl complaining of abdominal pain in whom a pregnancy slide test was slightly positive was operated upon for a left side ovarian tumour weighing 1900 g

Histological examination Endodermal sinus tumour combined with embryonal carcinoma and dysgerminoma.

Serum AFP concentration: 2560 µg/l.

Urine AFP concentration: 34 µg/l.

Extragonadal Tumours

1 *Mediastinal Region*

Case 18 A 5 -year-old man. X-ray examination of the chest showed a tumour mass in the anterior mediastinum. At explorative thoracotomy an inoperable tumour of orange-size was found. The patient died 3 month later of respiratory insufficiency Autopsy showed a large tumour in the mediastinum, extending into the lungs.

Histological examination Embryonal carcinoma with itelline components (vacuolated network) (Fig. 5 C)

Serum AFP concentration 4080 µg/l at operation and after one month 29 000 µg/l.

Case 19 A 21-year-old man who for several months had increasing respiratory symptoms. By X-ray examination at the chest, an anterior mediastinal tumour was found. Explorative thoracotomy showed an inoperable cystic tumour in the mediastinum, invading most of the right lung.

Histological examination Teratocarcinoma with several areas of itelline components (endodermal sinus tumour structures and vacuolated network) and germinoma components (Fig. 5 A and B)

Serum AFP concentration 390 µg/l.

Case 20 A 28-year-old man was admitted because of increasing respiratory insufficiency X-ray examination of the chest showed a large tumour in the anterior mediastinum. Explorative thoracotomy showed an inoperable necrotic cystic tumour about 15 cm in diameter and invasion into the right lung. No tumour was found in the testis.

Histological examination Severe necrotic embryonal carcinoma.

Serum AFP concentration 3750 µg/l.

2. *Retroperitoneal Region*

Case 21 A 27-year-old man was admitted because of acute abdominal pain. At operation, a primary inoperable retroperitoneal tumour was found close to the left kidney No tumour was found in the testis or in the liver

Histological examination: Polycystic teratoma with foci of endodermal cysts and a few endodermal sinus structures.

Serum AFP concentration 465 µg/l before operation and 8200 µg/l seven weeks later

Case 22 A 23-year-old man was admitted because of an abdominal tumour At operation, an inoperable retro-peritoneal tumour was found close to the pelvis region of the left kidney No primary testis tumour was found.

Histological examination Teratocarcinoma with a marked vitelline component (endodermal sinus structure vacuolated network and labyrinthine structure) (Fig. 8)

Serum AFP concentration 1600 µg/l.

3 *Sacrococcygeal Region*

Case 23: A 12-month-old girl was admitted because of a sacrococcygeal tumour appearing as a swelling in the gluteal region. During the last month before admission, the patient had been treated repeatedly for urinary infection. Rectal exploration and urography confirmed the tumour diagnosis. Tumour and lymph gland biopsy from the right inguinal region showed tumour tissue.

Histological examination Endodermal sinus tumour of the sacrococcygeal region. (Fig. 6 B)

Serum AFP concentration: 169.000 µg/l.

Case 24 A 19-month-old girl who 5 months previously was operated upon for a dermoid cyst in crena ani was readmitted to hospital because of a left inguinal hernia. At operation, a subcutaneous tumour measuring 2 cm in diameter and situated below the Fallopian ligament was removed.

Histological examination Typical endodermal sinus tumour (Fig 6 A) Serum AFP concentration was 7000 µg/l before operation and one week later it was 7100 µg/l. (see Fig. 2) Accordingly explorative laparotomy was performed and a primary inoperable sacrococcygeal tumour was found. Two months after operation, the serum AFP concentration was 44.300 µg/l. By immuno-fluorescence microscopy a marked staining of the hyaline globules was found. The AFP concentration in tumour tissue homogenate was 3.4 µg per g tissue



Fig. 5 Endodermal sinus tumours of the anterior mediastinum showing A. and B. Endodermal sinus structures and microcysts. Serum AFP 390 μg/l. Case 19 (21-year-old man) H.E., × 210 C. Vacuolated network. Serum AFP 4080 μg/l. Case 18 (32-year-old man) H. E., × 84

At operation, a right ovarian tumour (size 18 × 12 × 10 cm) and a left ovarian tumour (size 10 × 7 × 4 cm) were removed. Histological examination Tumour of combined type (endodermal sinus tumour associated with choriocarcinoma and dysgerminoma) (Fig. 7)

Serum AFP concentration a few days after operation 950 µg/l (see Fig. 2)

Urine AFP concentration 50 µg/l.

Urine HCG concentration 87000 IU/l.

Case 16 A 17-year-old girl in whom slide test on urine for pregnancy was positive was operated upon for an inoperable left side solid ovarian tumour with a few cystic areas measuring about 20 × 15 × 15 cm.

Histological examination Dysgerminoma with areas of typical endodermal sinus tumour

Serum AFP concentration 14 days after operation 7100 µg/l.

Urine AFP concentration 70 µg/l.

Case 17 A 14-year-old girl complaining of abdominal pain in whom a pregnancy slide test was slightly positive was operated upon for a left side ovarian tumour weighing 1900 g.

Histological examination Endodermal sinus tumour combined with embryonal carcinoma and dysgerminoma.

Serum AFP concentration 2360 µg/l.

Urine AFP concentration 34 µg/l.

Extragonadal Tumours

1 *Mediastinal Region*

Case 18 A 32-year-old man. X-ray examination of the chest showed a tumour mass in the anterior mediastinum. At explorative thoracotomy an inoperable tumour of orange-size was found. The patient died 3 month later of respiratory insufficiency Autopsy showed a large tumour in the mediastinum, extending into the lungs.

Histological examination Embryonal carcinoma *with vitelline components (vacuolated network)* (Fig. 5 C)

Serum AFP concentration: 4080 µg/l at operation and after one month 29.000 µg/l.

Case 19 A 21-year-old man who for several months had increasing respiratory symptoms. By X-ray examination at the chest, an anterior mediastinal tumour was found. Explorative thoracotomy showed an inoperable cystic tumour in the mediastinum, invading most of the right lung.

Histological examination Teratocarcinoma with several areas of vitelline components (endodermal sinus tumour structures and vacuolated network) and germinoma components (Fig 5 A and B)

Serum AFP concentration 390 µg/l.

Case 20 A 28-year-old man was admitted because of increasing respiratory insufficiency X-ray examination of the chest showed a large tumour in the anterior mediastinum. Explorative thoracotomy showed an inoperable necrotic cystic tumour about 15 cm in diameter and invasion into the right lung No tumour was found in the testis.

Histological examination Severe necrotic embryonal carcinoma.

Serum AFP concentration 5750 µg/l.

2. *Retroperitoneal Region*

Case 21 A 27-year-old man was admitted because of acute abdominal pain. At operation, a primary inoperable retroperitoneal tumour was found close to the left kidney No tumour was found in the testis or in the liver

Histological examination Polycystic teratoma with foci of endodermal cysts and a few endodermal sinus structures.

Serum AFP concentration 465 µg/l before operation and 8200 µg/l seven weeks later

Case 22 A 23-year-old man was admitted because of an abdominal tumour At operation, an inoperable retro-peritoneal tumour was found close to the pelvis region of the left kidney No primary testis tumour was found.

Histological examination Teratocarcinoma with a marked vitelline component (endodermal sinus structure vacuolated network and labyrinthine structure) (Fig. 8)

Serum AFP concentration: 1600 µg/l.

3 *Sacrococcygeal Region*

Case 23 A 12-month-old girl was admitted because of a sacrococcygeal tumour appearing as a swelling in the gluteal region. During the last month before admission, the patient had been treated repeatedly for urinary infection. Rectal exploration and urography confirmed the tumour diagnosis. Tumour and lymph gland biopsy from the right inguinal region showed tumour tissue.

Histological examination Endodermal sinus tumour of the sacrococcygeal region. (Fig. 6 B)

Serum AFP concentration: 169 000 µg/l.

Case 24 A 19-month-old girl who 5 months previously was operated upon for a dermoid cyst in crena ani was readmitted to hospital because of a left inguinal hernia. At operation, a subcutaneous tumour measuring cm in diameter and situated below the Fallopian ligament was removed.

Histological examination *Typical endodermal* sinus tumour (Fig 6 A) Serum AFP concentration was 7000 µg/l before operation and one week later it was 7100 µg/l. (see Fig 2) Accordingly explorative laparotomy was performed and a primary inoperable sacrococcygeal tumour was found. Two months after operation, the serum AFP concentration was 44.300 µg/l. By immuno-fluorescence microscopy a marked staining of the hyaline globules was found The AFP concentration in tumour tissue homogenate was 3.4 µg per g tissue.

Fig. 7. Endodermal sinus tumour component of combined germ cell tumour of the ovary. Serum AFP 550 μg/l. Case 15 (20-year-old patient). H.E., × 210

Fig. 8. Retroperitoneal teratocarcinoma showing a large vitelline component with endodermal sinuses and labyrinthine structures. Serum AFP 1600 μg/l. Case 22 (23-year-old man). H.E., × 525

Fig. 9. Indirect immunofluorescence AFP staining of the tumour from case 9. (Embryonal carcinoma of the testis with a few endodermal sinus structures). Positive staining of the cells lining the endodermal sinuses is seen. × 430.

TABLE 3 *Cases Reported in the Literature with a Specific Histology (Endodermal Sinus Tumour Component) and Quantitation of Serum AFP*

Authors	Gonadal sites			Extragonadal sites					Total
	Infant testis	Adult testis	Ovary	Mediastinum	Retroperitoneum	Sacrococcygeal region	Pineal region	Infant vagina	
Ballas (1972)			1						1
Wilkinson et al.[33] (1973)			1						1
Tschudy et al.[37] (1973)						1			5
Ballas (1974)		5	3						6
Itoh et al.[13] (1974)	2		4	1		1		1	9
Talerman et al.[35] (1974)	1§								1
Talerman & Haije[3] (1974)		6	2	1	1				10
Scully & McNeely (1974)							1		1
Present series	3	10	3	3	3	2			24

Included in *Ballas*

§ Included in the present series (case 1)

with tumour size. After complete removal of the tumour AFP disappears rapidly from serum, the half life being about 5 days, as seen in Fig. 2 (case 1) Therefore, quantitative determination of AFP in serum after operation is a useful prognostic parameter

Adult testis tumours (Fig 4 (cases 4–14)) producing AFP are very rarely pure, endodermal sinus tumours (26) usually they are 'teratocarcinomas' or embryonal carcinomas with vitelline elements or even seminomas associated with endodermal sinus tumour An increased AFP concentration, especially the very high values, seems to indicate a poor prognosis and perhaps metastases. It may therefore be important to measure serum AFP concentration before operation in all patients with testicular tumours.

Ovarian vitelline tumours, (Fig. 7 (case 15–17)) are usually seen in young women as histologically pure, endodermal sinus tumours, in some cases combined with choriocarcinoma and/or dysgerminoma. The AFP concentration in serum is high and the prognosis is very poor In all the three cases described in this study the urine AFP concentration was found to be increased while the urinary albumin excretion was not increased The mechanism behind this selective excretion of AFP in urine is not clear Assessment of AFP and human chorionic gonadotrophin (HCG) in serum and urine after operation may be used as markers for re-occurrence of the different tumour cells (6 8) As seen in case 15 (Fig. 2) a marked fall in the AFP concentration is seen during the postoperative period but a marked resynthesis of AFP takes place about two months later

Extragonodal Tumours producing AFP

Germ cell tumours of *the anterior mediastinum*, (Fig. 5 (cases 18–20)) occur almost exclusively in males and are very malignant tumours. The rapid growth of these tumours can be registered by the marked increase in the serum AFP concentration, as seen in case 18. *Retroperitoneal* germ cell tumours (cases 21 22) are also very malignant.

Germ cell tumours of *the sacrococcygeal region* (Fig 6 (cases 23–24)) are found mainly in girls (74 per cent) under two years of age. In case 24 (Fig. 2) a rapid increase in serum AFP is seen.

As seen in Table 3 it has been observed in several studies in which a specific histology is used that serum AFP is elevated in endodermal sinus tumours or tumours with a marked vitelline component. Even in a case of endodermal sinus tumour in the region of *the pineal gland* a post mortem test for serum AFP was found to be positive (19) Also in a case of endodermal sinus tumour of *the infant vagina* the serum AFP has been found to be elevated (12)

A raised serum AFP concentration in patients with teratocarcinoma was first, and independently described by *Abelev et al.* (1967) and *Masopust et al.* (1968) These observations have been confirmed in other studies (2 3 6, 7 16 20) and serum AFP estimations have also been used as an indicator for therapy (6 7 8, 13)

Results of the recent studies, combined with *Teilums* concept of the endodermal sinus tumour however stress the importance of using a specific histology in future studies. Quantitation of AFP in serum and urine might thus serve to aid the histopathologists in attempts at establishing new functional criteria for the classification of the respective tumours (3 5 14)

Also the immunofluorescence staining technique for demonstration the AFP synthesis in the tumour tissue may be of importance for the histological interpretation. The AFP synthesis in germ cell tumours containing only a few vitelline components seems to take place e.g In the cells lining the endodermal sinuses, as seen in Fig 9 whereas areas with solid embryonal carcinoma show a negative staining reaction. However further immunofluorescence studies should be carried out.

Application of a sensitive AFP quantitation test will be of important for the clinical diagnosis, estimation of prognosis and for the clinical follow-up of patients with these tu-

mours, as seen in Fig 2. If the AFP level in serum is related to the tumour mass present the AFP quantitation can be a useful tool for selection and monitoring of patients as well as for radiotherapeutic and chemotherapeutic regimes. Furthermore, a persistently elevated AFP in serum after operation indicates metastases.

A selective blood screening for AFP should be carried out in all patients with testis tumours and, after operation also in all patients in whom tumours are classified as embryonal carcinoma or teratocarcinoma. As seen in Fig 2 the half life of serum AFP is about 5 days and thus, elevated blood AFP levels can still be demonstrated several days or weeks after operation.

Dr *H E Christensen* Dr *S Fischer* Dr *K Mauritsen*, Dr *B Mogensen*, Dr *Hemming Poulsen* Dr *A. Sell* Dr *H Søgaard* Dr *J V Thorborg*, Dr *I Tygstrup* and Dr *M Vetner* are gratefully acknowledged for supplying us with tumour tissue and blood samples. This study was supported by grants from the *Danish Cancer Society* The technical assistance of Miss *Kirsten Larsen* is highly appreciated.

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THE ROLE OF THE PARATHYROIDS FOR THE ADAPTATION TO A LOW CALCIUM INTAKE

3 The Long-Term Effect of Parathyroidectomy on the Adaptation to a Low Calcium Intake in Adult Rats with Special Reference to Plasma Calcium Bone Tissue and Adrenal Glands

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Ahlgren, O & Larsson, S. E. The role of the parathyroids for the adaptation to a low calcium intake. 3 The long-term effect of parathyroidectomy on the adaptation to a low calcium intake in adult rats with special reference to plasma calcium, bone tissue and adrenal glands. Acta path. microbiol. scand. Sect. A, 83 590–602, 1975.

One-year-old selectively parathyroidectomized rats responded with a plasma calcium reduction to below 4 1 mEq./l in 86 per cent at a normal and in 100 per cent at a low dietary calcium intake. Among the former 17 per cent normalized their plasma calcium level within 8 weeks and another 40 per cent between the 8th and 27th week of observation. Among the latter, only 10 per cent showed a normalization and first after the long-term period of observation. On the normal level of dietary calcium the parathyroidectomized animals with persistently reduced plasma calcium showed a significantly increased bone mass which was paradoxical in view of their inability of adaptation. On the low level of dietary calcium, a normal bone resorptive activity was maintained despite parathyroidectomy possibly through the action of increased levels of 1,25-dihydroxycholecalciferol provoked by the profoundly reduced plasma calcium. This was, however insufficient for adaptation and no osteoporosis developed. For the adaptation to a reduced calcium intake skeletal calcium reserves had to become mobilized through the action of the parathyroids with resulting osteoporosis. This was brought about by increased mobilization of skeletal calcium with resulting osteoporosis, a phenomenon which could be seen also among the parathyroidectomized animals on a low dietary calcium and with a normalized plasma calcium level. This osteoporosis was similar to that found in the calcium deficient intact animals. The described bone changes were progressing in character and there was no major influence by any eventual effect on bone growth. At histological and morphometric analyses of the adrenal cortex no apparent changes were found after parathyroidectomy

Key words Calcium deficiency calcium metabolism parathyroidectomy osteoporosis osteopetrosis adrenal glands.

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In previous short term investigations, one year-old selectively parathyroidectomized rats were found to have lost their ability to adapt themselves to a reduced calcium intake over a period of eight weeks (*Larsson & Ahlgren*

1975 a, *Ahlgren & Larsson* 1975 a). This was due to an inability to mobilize skeletal calcium to the blood in the absence of or at a low level of parathyroid hormone despite an adequate supply of dietary vitamin D (*Larsson & Ahlgren* 1975 a). While intact animals developed calcium-deficiency osteoporosis, parathyroidectomized animals did not. Intestinal net absorption of calcium, which is regulated by the action of the active metabolite of vitamin D 1,25-dihydroxycholecalciferol (*Holick et al.* 1971 *Myrtle & Norman* 1971) was not impaired after parathyroidectomy neither at a normal nor at a low intake of dietary calcium (*Ahlgren & Larsson* 1975 a). Urinary calcium excretion was reduced because of the reduced plasma calcium level. It was concluded that the vitamin D regulated intestinal calcium absorption was not primarily influenced by the activity of the parathyroid glands. Mobilization of skeletal calcium through the action of parathyroid hormone was found to be necessary for the adaptation to a low calcium intake in the intact adult animal besides vitamin D. The occurrence of parathyroid hyperplasia (*Samatiloglou & Larsson* 1972) has been demonstrated during the development of calcium deficiency osteoporosis in adult rats (*Larsson* 1969).

The results of our previous studies (*Larsson & Ahlgren* 1975 a, *Ahlgren & Larsson* 1975 a) seem to indicate that the reported action of vitamin D metabolites as stimulators of bone resorption (*Trummel et al.* 1969 *Raisz et al.* 1972) is present only in concert with parathyroid hormone in a physiological situation *in vivo*. From studies of young mice it has been reported that 1,25-dihydroxycholecalciferol is one of the most potent substances known that stimulates bone resorption as examined with an *in vivo* / *in vitro* method of experimentation (*Reynolds et al.* 1973). Since the substance was effective with no parathyroid hormone in the culture medium there is the possibility that increased bone resorption might be induced by low calcium intake also in parathyroidectomized animals although not to the same extent as in intact animals. The present *long-term* experiment was undertaken in order to examine this possibility further.

The maintenance of the plasma calcium level after parathyroidectomy is apparently dependent upon the level of dietary calcium intake but also upon other factors as functioning accessory parathyroid glands as discussed in our previous work (*Larsson & Ahlgren* 1975 a). With the time after parathyroidectomy there seemed to be an increasing efficiency of the remaining adaptory mechanisms to restore the plasma calcium level. In view of the reported increase in intestinal net absorption of calcium (*Ahlgren & Larsson* 1975 a) either an increased synthesis of active vitamin D metabolites or a selective increased accumulation of 1,25-dihydroxycholecalciferol in the intestinal mucosa (*Favus et al.* 1974) may well be operative compensatory to the induced reduction of the parathyroid hormone levels. The present *long-term* investigation aims at examining the possible progress or reversibility of the described *short-term* effects of parathyroidectomy on the plasma calcium level, the body weight, the adrenal cortex, the calcium accretion rate by bone and the bone *mass at a low* and a normal level of dietary calcium intake. A subsequent study of the same animals will deal with the effects on the calcium metabolism.

MATERIAL AND METHODS

Experimental Animals and Housing

A detailed description has been given in our previous short-term study (*Larsson & Ahlgren* 1975 a) which is referred to. Out of a total of 137 sterile bred male rats of the Sprague-Dawley strain[1] 40 were used in the short-term series. Remaining surviving animals were used in the present, long-term series comprising one observation period of 5 months. All animals were stated to be 12 to 12½ months old at the start of the experiment and their initial body weights were 515–645 grams.

Experimental Diets

The low calcium diet of General Biochemical Inc., Chagrin Falls, Ohio, was used as a basic

[1] Obtained from Møllegaard Hansens Avlslaboratorium A/S, Ejby, Denmark.

ration. A detailed description of this diet has been given elsewhere (*Larsson* 1969). The diet contained 0.04 per cent Ca and 0.70 per cent P with a Ca:P ratio of 1:17.5 and was supplied with vitamin D_3 600 I.U. per 100 g diet. The amount of Mg in the diet was 0.20 per cent.

The normal calcium diet consisted of the low calcium test diet supplemented with $CaCO_3$, this diet thus containing a total amount of 1.2 per cent Ca and 0.70 per cent P. The Ca:P ratio was 1:0.53.

Diets** and deionized water were given *ad libitum*.

Parathyroidectomy (PTE)

This was performed in the two series in a total of 95 animals as described previously (*c.f. Larsson & Ahlgren* 1975 a). Completeness of removal was checked by analyses of plasma calcium (see Plasma Sampling). Only animals which showed a post-operative fall in the plasma calcium level to below 4.1 mEq./l were accepted as PTE animals (*c.f. Johansson & Segerström* 1972). With exception of the animals used in the short-term series the following groups of animals were obtained for the present series depending upon treatment and plasma calcium level.

Experimental animals: PTE animals were divided into the following groups of the long-term series.

PTE — Ca: twenty-one PTE rats supplied the low calcium diet showing a consistently low plasma calcium level.

PTE + Ca: six PTE rats supplied the normal calcium diet showing a consistently low plasma calcium level.

PTE_N — Ca: three PTE rats given the low calcium diet showing a normalization of the plasma calcium level after an initial reduction.

PTE_N + Ca: eight PTE rats supplied the normal calcium diet having normalized their plasma calcium level after an initial reduction.

Intact animals were randomly divided into the following groups.

— Ca: seven animals supplied the low calcium diet.

+ Ca: eight animals given the normal calcium diet thus constituting the control group.

Body Weight, Food Consumption and Stools

All the animals were weighed at 14 days intervals during the experimental period. Food consumption and the amount of stools were determined during the last 3 days of the experiment when the animals were kept isolated in metabolic cages.

Plasma Sampling

From each animal, approximately 0.5–1.0 ml of blood was taken by cardiac puncture under light ether anaesthesia 2 days, 4, 6 and 14 weeks after the start of the experiment and 4, 24, 48 and 72 hours before sacrifice of the animal. Finally blood was collected at the time of sacrifice which was performed by bleeding the animal to death from the femoral artery under ether anaesthesia. The collected blood was centrifuged at 3,000 r.p.m. for 15 minutes and the supernatant plasma was pipetted into acid washed test tubes and kept at −20° C pending subsequent analyses.

The following procedures were performed as described previously (*Larsson & Ahlgren* 1975 a):

Determination of Plasma Calcium

Determination of Moisture, Organic and Inorganic Matter of the Tibia

Preparation of Standardized Undecalcified Bone Sections of the Femur

Microradiography

Measurement of the Cortical Thickness, the Outer and Inner Diameters of the Mid-shaft of the Femur

Morphometric Analyses of the Relative Amount of Spongious Bone Tissue in the Distal Femur

Determination of the Calcium Accretion Rate for the Tibia

Weight of the Adrenal Glands

Preparation of Histological Sections of the Adrenals

Morphometric Analyses of the Relative Thickness of the Adrenal Cortex

Control of the Completeness of Parathyroid Removal

The Volume of the Tibia

This was determined by submerging the hydrated whole bone in distilled water at a temperature of 4° C. The volume was calculated as the weight of water displaced by the bone (*c.f. Robinson & Elliott* 1957).

Statistical Treatment of Data

Data applying to the different groups were compared in pairs and subjected to the Student's t-test. The hypothesis of no difference between groups was tested with a 5 per cent significance level.

* Here and in the following text the figures calculated on a dry weight basis are given.

** Obtained from Ewos Co., Södertälje, Sweden.

TABLE 1 *The Distribution of the Whole Experimental Material According to Treatment and Plasma Calcium Level*

Group of animals	Short-term series. Number of animals in respective group at different periods after PTE					Long-term series			Remaining (for another exp.)
	Start	2 days	4 weeks	8 weeks	Sacrificed	14 weeks	27 weeks	Sacrificed	
+ Ca	20	19 (1)	19	19	5	14	14	8 (1)	5
− Ca	22	22	21 (1)	21	3	16	16	7 (4)	4 (1)
PTE + Ca	42	36	32	30	9 (1)	17 (2)	10	6 (4)	−
PTE − Ca	45	42 (1)	41 (1)	40 (1)	7 (1)	32	29	21 (5)	5
PTE_X − Ca	−	−	−	−	−	−	3	5	−
PTE_Y + Ca	−	−	4	6	6	1	8	8	−
PTE_{XY} + Ca	−	6	8	6	6	−	−	−	−

For explanation, see page 5 Ten animals died during the parathyroidectomy procedure Figures within brackets denote animals which died during the experiment.

RESULTS

Short Term and Long Term Series

Mortality

Seventeen deaths occurred in the two series among a total of 127 animals which were subjected to repeated cardiac punctures for blood collection. These deaths were caused by haemopericardium or pneumothorax which occurred in connection with the cardiac puncture and showed a relatively even distribution among the various groups of animals (Table 1) Out of a total of 95 parathyroidectomies performed there were 10 deaths which occurred during the operation. No deaths were caused by postoperative tetany

Among the animals constituting the long term series there were another 2 deaths, one in the group of PTE rats fed the low calcium diet and one in the group of PTE rats supplied the calcium supplemented diet. These deaths occurred among animals showing reduced plasma calcium level but were not caused by hypocalcaemic tetany but by some unrelated disease. The dead animals were excluded. All the other animals were in good health.

Plasma Calcium

The whole experimental material with exception of the 10 animals which died at parathyroidectomy was primarily divided at random into intact animals supplied the normal and low calcium diet, respectively and into PTE animals likewise given the normal and low calcium diet, respectively (Table 1) Thereafter the main groups of PTE animals were divided into sub-groups depending upon whether or not their plasma calcium showed a concentration below 4 1 mEq./l which was the limit used for determination postoperatively which animals should be accepted as PTE animals. As shown in Table 1 and Fig 1 the PTE animals had either a persistently low plasma calcium level below this limit or were able to normalize their plasma calcium concentration during the run of the experiment. Among a total of 42 animals subjected to PTE and a normal calcium intake, 36 (86 per cent) responded with a plasma calcium reduction which fulfilled our criterion for a verified parathyroidectomy Six of these animals (17 per cent) normalized their plasma calcium level within 8 weeks and were included in the short term series as were those with a non-verified parathyroidectomy

In the long term series, out of 20 PTE animals fed the normal calcium diet and showing a persistently reduced plasma calcium level during the first 6 weeks of observation, one showed normalized plasma calcium values after 14 weeks and another seven animals after 27 weeks. Thus, 40 per cent of PTE animals with a normal calcium intake showed

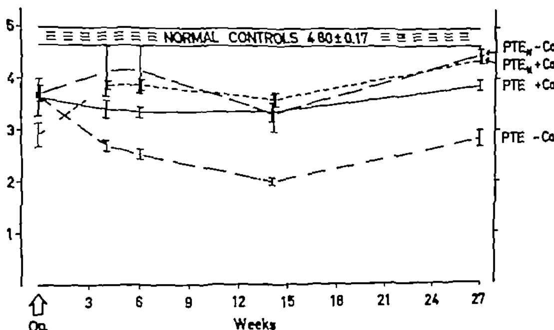


Fig 1 Plasma calcium of the normal control group and the various experimental groups during the experimental period of 27 weeks. The bars represent the standard error of the mean. The values obtained for the two groups of intact animals which were kept on normal (+ Ca) and low (— Ca) dietary calcium, respectively did not differ significantly PTE = parathyroidectomy PTE_N = PTE animals showing a normalized plasma calcium level.

a normalization of their plasma calcium level after a long term period of observation. While none of the PTE animals supplied the low calcium diet was able to normalize the plasma calcium level within 8 weeks, 3 animals out of 32 included in the long-term series (9 4 per cent) showed normalized plasma calcium values after 27 weeks of observation (Table 1)

Plasma calcium of the final groups of animals of the long term series is shown in Fig 1 As regards intact animals there was no significant difference between rats fed the low calcium diet and those supplied the calcium supplemented diet. Among the PTE animals with a persistently reduced plasma calcium level those with a normal calcium intake showed values varying between 2.70 and 4 10 mEq /l after 4 6, 14 and 27 weeks while the corresponding values for those with a low calcium intake varied between 1.80 and 3.75 mEq /l. After 27 weeks of observation, the mean value of the former group of animals was 18.8 per cent and that of the latter group 39 6 per cent lower than the corresponding value of the normal control animals, the differences being highly significant. While the plasma calcium values 2 days postoperatively did not differ significantly between the groups of PTE animals fed the normal and low calcium diet, respectively the latter group showed a further decrease to a level that was approximately 25 per cent lower than that of the former from 4 weeks and on. The group of PTE_N animals supplied the normal calcium diet showed still significantly lower values than normal although these animals had "normalized" their plasma calcium level according to our criterion.

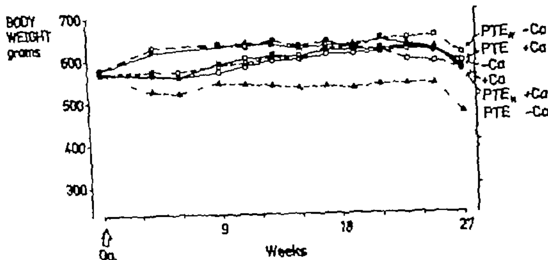


Fig 2 Mean body weights of the normal control group (+ Ca) and the various experimental groups.

Long-Term Series

Body Weight

Intact animals showed almost constant or slightly increased body weight during the experimental period, no matter whether the animals were fed the low or normal calcium diet (Fig. 2). At the end of the experimental period all groups of animals showed a slight to moderate body weight decrease which occurred mainly during the period when the animals were kept in metabolic cages. As regards the PTE animals supplied the normal calcium diet almost constant body weights were noted both in animals with persistently low plasma calcium and in those with normalized plasma calcium level. PTE animals fed the low calcium diet and showing a very low plasma calcium level maintained a constant body weight after a slight initial reduction. The loss in body weight at the end of the experimental period was somewhat more pronounced in this group of animals than in the other experimental groups. In contrast, PTE animals supplied the low calcium diet and showing a normalization of the plasma calcium level increased their body weight slightly during the last half of the experimental period.

Composition of the Tibia

The inorganic contents of the whole tibia calculated per wet weight showed lower ($p<0.001$) values in the calcium-deficient intact animals compared with those obtained for the normal controls (Fig 3). The lower contents of inorganic matter corresponded to higher ($0.005<p<0.001$) contents of moisture while the contents of organic matter showed no significant difference. The group of PTE animals supplied the normal calcium diet and having persistently reduced plasma calcium showed higher contents of inorganic matter than noted in the normal control group, the difference being almost significant ($0.05<p<0.10$) and lower contents of organic matter ($0.025<p<0.05$) while water contents were somewhat increased ($0.05<p<0.10$). The values for the inorganic contents of the tibia obtained for the PTE animals fed the low calcium diet and showing reduced plasma calcium were higher ($p<0.001$) and organic contents lower ($0.05<p<0.10$) as well as water contents ($0.025<p<0.05$) than those of the intact animals supplied the low calcium diet. The values obtained for the PTE animals fed the low calcium diet did not differ significantly from those obtained for the normal controls. On the other hand, for the group of calcium-

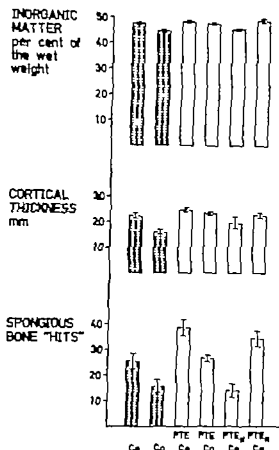


Fig 5 The inorganic matter of the tibia, the cortical thickness of the mid-shaft of the femur and the number of spongious bone "hits" of the distal femur metaphysis in the normal control group (+ Ca) and the various experimental groups. Note the significant reduction of bone mass in the calcium-deficient intact animals (— Ca) and in PTE$_N$ —Ca animals. While the groups PTE —Ca and PTE$_N$ + Ca showed no significant difference from normal, PTE + Ca showed significantly *increased* bone mass.

deficient PTE animals with normalized plasma calcium level lower ($0.005<p<0.01$) values were obtained for the contents of inorganic matter which did not differ significantly from those obtained for the calcium-deficient intact animals. The values obtained for the contents of organic matter and moisture showed no significant difference between these groups.

Cortical Thickness Outer and Inner Diameters of the Mid-Shaft of the Femur and Volume of the Tibia

Compared with the normal control animals, calcium-deficient intact animals showed lower ($p<0.001$) values for the cortical thickness with higher ($0.001<p<0.005$) values for the inner diameter and no significant difference as regards the outer diameter (Table 2) Calcium-deficient PTE animals having reduced plasma calcium level showed higher ($p<0.001$) values for the cortical thickness than those of the calcium-deficient intact animals and the values did not differ significantly from those of the normal controls. Similar values were obtained for the PTE animals fed the normal calcium diet and having reduced plasma calcium. The group of calcium-deficient PTE animals showing normalized plasma calcium level had values for the cortical thickness which were not significantly different from normal, higher ($0.05<p<0.10$) than those of the calcium-deficient intact animals and lower ($0.05<p<0.10$) than those of the calcium-deficient PTE animals with persistently reduced plasma calcium.

As regards the outer and inner diameters, a reduced cortical thickness was associated with an increased inner diameter with no major change of the outer diameter However at a normal calcium intake PTE animals with reduced plasma calcium showed larger ($0.05<p<0.10$) outer diameter than intact animals. Among the hypocalcemic PTE animals, those with a normal calcium intake showed higher values for the outer diameter than those of the corresponding calcium-deficient animals, this difference also being almost significant ($0.05<p<0.10$)

The volume of the whole tibia (Table 2) showed no significant difference between intact calcium-deficient animals and control animals nor between intact and PTE animals either at a normal or at a low calcium intake The values obtained for the PTE animals with reduced plasma calcium were higher ($0.025<p<0.02$) for those with a normal

TABLE 2. *The Cortical Thickness the Outer and Inner Diameters of the Mid-Shaft of the Femur and the Volume of the Tibia in the Normal Control Group and the Various Experimental Groups*

Group	Femur Cortical thickness mm	Femur Outer diameter mm	Femur Inner diameter mm	Tibia Volume cm³
Intact controls, + Ca	2.29 ± 0 11	5 45 ± 0.07	3 16 ± 0.09	0.64 ± 0.013
Intact animals, − Ca	1.58 ± 0.10	5 45 ± 0.09	3.88 ± 0.15	0.63 ± 0.018
PTE animals, + Ca	2.48 ± 0.07	5.65 ± 0.08	3 17 ± 0.09	0.66 ± 0.022
PTE animals, — Ca	.34 ± 0.03	5 42 ± 0 05	3.07 ± 0 04	0 62 ± 0.008
PTE_x animals, — Ca	1.97 ± 0.23	5.30 ± 0.13	3.33 ± 0.23	0.63 ± 0.025
PTE_x animals, + Ca	..33 ± 0.09	5.53 ± 0.10	3.20 ± 0 08	0.66 ± 0.016

Mean values and standard errors of the mean are given. Note the reduction in cortical bone through endosteal resorption in calcium-deficient intact and PTE_x animals.

calcium intake compared to those with a low calcium intake, PTE animals with normalized plasma calcium showed values which were similar to those of the normal controls.

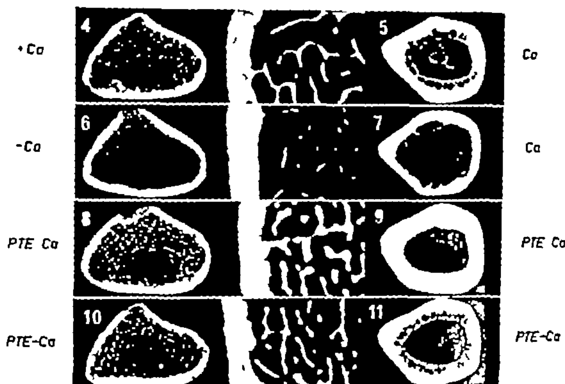
Morphometric Analyses of the Relative Amount of Spongious Bone Tissue

comparison with the values obtained for ormal control animals those of the cal- -deficient intact animals were lower $< p < 0.025$) (Fig 3) At a normal cal- intake, higher ($0.01 < p < 0.02$) values obtained for the PTE animals with per ntly low plasma calcium level compared those of the intact controls. Also at the calcium intake higher ($p < 0.001$) values e obtained for the PTE animals with re d plasma calcium compared with those he intact animals. In fact, this group of ium-deficient PTE animals showed values ch were not significantly different from e of the intact controls fed the normal cium diet. On the other hand, at a low cium intake PTE animals with normalized sma calcium showed lower ($0.001 < p <$ 05) values than the corresponding group th persistently reduced plasma calcium. At a mal calcium intake, PTE animals showed milar values regardless of whether the plas- a calcium level was normalized or not.

Microradiography

Microradiographs of standardized undecal cified cross sections of the distal femur meta physis and the femoral mid-shaft had a simi lar appearance in all animals of the normal control group showing evenly mineralized cortical and trabecular bone (Figs. 4 and 5) In the calcium-deficient intact animals, the bone trabeculae of the metaphyseal bone were considerably reduced in number and con- spicously thinner than those of the normal controls and the cortex of the diaphyseal bone showed a reduced thickness (Figs. 6 and 7) The remaining cortical and spongious bone showed a normal degree of mineralization. PTE animals supplied the normal calcium diet and having a reduced plasma calcium level showed definitely thicker bone trabeculae and cortical bone than normal (Figs. 8 and 9)

Generally the changes of the cortical bone were as evident as those of the spongious bone and involved the inner part of the cortex mainly with no major change of the outer diameter of the bone. In the normal control animals the inner part of the diaphyseal cortex consisted of dense bone and also mar row cavities which in some areas were sepa- rated by bone trabeculae (Fig 5) While calcium-deficient intact animals showed al- most complete disappearance of this inner part of the bony cortex it was transformed into thickened thoroughly dense bone in the



Figs. 4–11 Microradiographs of undecalcified cross sections of the distal femur metaphysis at linear magnifications of 20× and 50× (left) and of the mid-shaft of the femur at 20× (right). Note the occurrence of osteoporosis with loss of both spongious and endosteal cortical bone in the group of calcium-deficient intact animals (Figs. 6 and 7) and the increase in both spongious and cortical bone, (osteopetrosis) in the group of PTE + Ca animals (Figs. 8 and 9). The group of PTE — Ca animals showed no differences from normal (Figs. 10 and 11)

hypocalcemic PTE animals supplied the normal calcium diet (Figs. 6 and 7).

In the calcium deficient PTE animals with reduced plasma calcium level microradiographs showed both spongious and cortical bone with quite a normal appearance (Figs. 10 and 11). In comparison, an evident reduction was found of particularly the spongious bone in the calcium-deficient PTE animals which had normalized their plasma calcium level. No such changes were noted in the corresponding group of animals fed the normal calcium diet.

Calcium Accretion Rate by Bone

Intact animals showed higher ($0.025 < p < 0.05$) accretion rate of the tibia at a low intake of dietary calcium than at a normal intake (Fig. 12). PTE animals with reduced plasma calcium showed values which were not significantly different from those of the normal controls both at a normal and low calcium intake. Thus, while a low calcium intake resulted in significantly increased accretion rate in intact animals no such change occurred in calcium-deficient PTE animals with persistently reduced plasma calcium. In comparison, the corresponding group of PTE animals with normalized plasma calcium showed increased ($0.01 < p < 0.02$) calcium accretion rate comparable to those obtained for the calcium-deficient intact animals. PTE animals kept on the normal calcium diet and having a normalized plasma calcium level showed values which were not significantly different from those of the normal controls.

Effects of PTE on the Thyroid Gland

Histological examination of serial sections of the thyroid gland showed remaining para-

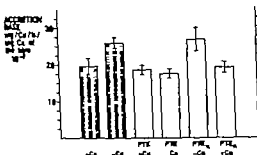


Fig. 12 Calcium accretion rate of the tibia in control (+ Ca) and experimental groups. Note the significant increase in the groups of — Ca, and PTE_M — Ca animals certainly secondary to still more increased bone resorption.

thyroids in 2 out of the total of 95 PTE animals. These two animals which did not show a reduction of their plasma calcium have been reported on previously (*Larsson & Ahlgren* 1975 a)

Except for a relatively small scar remaining at the site of the parathyroids after their removal, the thyroid had quite a normal appearance in all animals of the present series. No remaining parathyroid tissue was found.

Any eventual changes in the functional status of the C-cells could not be observed light microscopically

Effects of PTE on the Adrenal Glands

The wet weight of the two glands and the relative thickness of the adrenal cortex showed no significant differences between the normal control group and the various experimental groups. Frozen sections stained with Sudan Black B showed the adrenal cortex of a normal histological appearance. Within the series no noteworthy changes could be observed as to the relative thickness of the three cortical zones. A few animals seemed to be slightly more rich in lipids in the glomerular and fasciculate zones than ordinarily observed but the changes were quite small and not as evident as the corresponding changes reported on in the short term series (*Larsson & Ahlgren* 1975 a)

DISCUSSION

Our criterion for a verified parathyroidectomy was the same as that used by *Johansson & Segerström* (1972) for the same strain of rats i.e. a postoperative decrease of the plasma calcium level to 4 1 mEq /l or less. In the two series, a verified parathyroidectomy was achieved in as much as 86 per cent of rats fed the normal calcium diet and in 100 per cent of those supplied the low calcium diet (*Larsson & Ahlgren* 1975 a) Within 8 weeks, 17 per cent of the former normalized their plasma calcium level but none of the latter animals. Between the 8th and 27th week of observation, another 40 per cent of the parathyroidectomized animals with a normal calcium intake showed a normalization of their plasma calcium in the present study but only 10 per cent of those fed the low calcium diet. The normalization of the plasma calcium level might be due to the presence of supernumerary parathyroid glands in the neck. These are present in the rat in 30 per cent according to *Casewell & Fennell* (1970) but a frequency of as high as 80 per cent has also been reported (*Kenny* 1960) None of the animals of the present long term series showed any remaining parathyroid tissue within the thyroid gland nor were any accessory parathyroid glands detected at careful examination of the mediastinum with the aid of a dissection microscope and intravital staining. Such a method has been reported to be useful in locating parathyroid tissue in ectopic sites clinically (*Rosen et al.* 1974) However a negative result does not exclude the possibility of diffusely distributed functioning parathyroid tissue at aberrant sites which we were unable to detect with this procedure. In spite of these circumstances there were no difficulties of producing a state of hypoparathyroidism in the adult rats used in the present investigation contrary to the report by *Johansson & Segerström* (1972) from studies of growing rats.

Both at a low and at normal intake of dietary calcium an increasing number of animals was thus able to normalize their plasma

calcium level with increasing time after parathyroidectomy. However, at a low calcium intake only three out of forty three animals showed a normalization of their plasma calcium and this occurred first after 14 weeks from the time of parathyroidectomy. This was apparently brought about by mobilization of skeletal calcium through increased bone resorption. Thus, the bone tissue showed evident osteoporotic changes which were almost as pronounced as those observed in the calcium-deficient intact animals. Calcium accretion rate by bone was increased certainly secondary to the still more increased resorption of bone. Studies of the calcium metabolism (*Larsson* & *Ahlgren* 1975 b) showed also that these two groups of animals were comparable which strongly suggest that the adaptation, *viz.* normalization of the plasma calcium, was the result of hormonally active aberrant parathyroid tissue rather than increased levels of 1,25-dihydroxycholecalciferol.

On the other hand, at a normal calcium intake the group of parathyroidectomized animals with normalized plasma calcium showed data which were not comparable to those of the normal control group. Although calcium accretion rate by bone did not differ significantly the former group showed a tendency at an increase in the mineral contents of the whole bone and in the amount of spongious bone tissue, these differences being almost significant statistically. There was also a corresponding significant decrease in the organic constituents of the whole bone. Microradiographs showed thicker bone trabeculae and more dense bony cortex than in the normal controls.

The increase in bone mass was even more evident in the group of parathyroidectomized rats with normal calcium intake and showing a persistently *reduced* plasma calcium level. This is paradoxical in view of their inability of adaptation, *viz.* normalization of their plasma calcium. Since accretion rate by bone was normal the observed increase in bone mass must have been the result of lack of bone resorption secondary to the absence of parathyroid hormone. Although these animals showed a tendency at reduced net absorption of intestinal calcium, parathyroidectomized animals showed no disturbance of their ability to increase their intestinal net absorption of calcium upon reduction of the dietary calcium intake (*Ahlgren* & *Larsson* 1975 a). Urinary calcium excretion was lowered secondary to the low plasma calcium level. These findings thus demonstrate the importance of the mobilization of skeletal calcium, *i.e.* the physiological processes of bone resorption regulated by parathyroid hormone, for the maintenance of a normal plasma calcium level. While the increase in bone mass was more evident in parathyroidectomized animals with reduced plasma calcium than in those with normalized plasma calcium at a normal calcium intake there were no major differences as regards the calcium metabolism (*Ahlgren* & *Larsson* 1975 a). Both groups of animals appeared to show a state of hypoparathyroidism although it was less pronounced in the group with normalized plasma calcium. This group of animals showed, in fact, still significantly lower plasma calcium values than those of the normal control group. The values obtained for the intestinal net absorption of calcium did not seem to indicate any major change in vitamin D metabolism in these groups of animals.

The bone changes induced in the present study were progressing in character. The calcium-deficient intact animals showed a more advanced degree of osteoporosis than that observed in the short term investigation (*Larsson* & *Ahlgren* 1975 a) which is in agreement with previous reports from our laboratory (*Larsson* 1969). The calcium accretion rate by bone was still increased secondary to the even more increased bone resorption. The significant increase in bone mass found in the group of persistently hypocalcaemic parathyroidectomized rats with a normal calcium intake could not be observed with certainty in the corresponding group of our short-term series (*Larsson* & *Ahlgren* 1975a). Thus, the induced bone changes described in our short-term series did not become stationary later on but showed a steady progress with increasing time

None of the various groups of experimental animals showed any difference from normal as regards the volume of the whole tibia. This finding is in agreement with the report that calcification of bone in rats is constant after 6 months of age (*Henry & Kon* 1953) and also new bone formation and bone growth (*Bohr* 1968). The increase in spongious and cortical bone mass observed in the group of hypocalcaemic parathyroidectomized animals with normal calcium intake was also followed by increased bone volume which was significant in comparison only with that of the corresponding group of animals fed the low calcium diet. The latter animals showed lower body weight than the former but it was relatively constant after an initial reduction. The adrenal cortex and the thyroid showed a normal histological appearance in all groups of animals. There were no apparent nutritional disturbances which might have influenced the results except for the differences in dietary calcium intake. In the light microscope, we were not able to observe any eventual changes in the functional status of the C-cells of the thyroid.

As in our short-term study (*Larsson & Ahlgren* 1975 a) changes indicating any degree of osteoporosis did not occur in the group of calcium-deficient parathyroidectomized animals which showed persistently reduced plasma calcium level. The bone tissue as well as the calcium accretion rate by bone showed no differences from normal. These animals had a plasma calcium level that was still lower than that of the hypocalcaemic parathyroidectomized animals with a normal calcium intake although they showed a significantly higher intestinal net absorption of calcium and inorganic phosphate in plasma was highly elevated (*Ahlgren & Larsson* 1975 a). The absence of an expected increase in bone mass in this group of animals observed in the corresponding group of animals with normal calcium intake might conceivably be explained by an increased level of circulating 1,25-dihydroxycholecalciferol induced by the low plasma calcium concentration. This would then maintain bone resorption at a normal level. In young growing rats, a reduced level of circulating 1,25-dihydroxycholecalciferol has recently been reported after thyroparathyroidectomy and supplementation with L-thyroxine (*Favus et al.* 1974) contradictory to the conclusions arrived at above. This difference might well be explained by the different experimental model used by those investigators. The metabolism of vitamin D and its relation to plasma calcium and parathyroid activity is currently being investigated using a similar experimental material as in the present investigation.

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Acta path. microbiol. scand. Sect. A, 83 605–614 1975

THE ROLE OF THE PARATHYROIDS FOR THE ADAPTATION TO A LOW CALCIUM INTAKE

4 *The Long-Term Effect of Parathyroidectomy on the Adaptation to a Low Calcium Intake in Adult Rats with Special Reference to Calcium Metabolism*

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Larsson, S.-E. & Ahlgren, O The role of the parathyroids for the adaptation to a low calcium intake. 4 The long-term effect of parathyroidectomy on the adaptation to a low calcium intake in adult rats with special reference to calcium metabolism. Acta path. microbiol. scand. Sect. A, 83 605–614 1975

One-year-old selectively parathyroidectomized rats showed, on a normal dietary intake of calcium, phosphorus and vitamin D a reduction in plasma calcium to below 4.1 mEq./l in 86 per cent and on restricted calcium intake in 100 per cent. On the normal level of dietary calcium, normalization of plasma calcium occurred in 17 per cent within 8 weeks and in another 40 per cent between the 8th and 27th week of observation while on the low level only 10 per cent of the animals normalized their plasma calcium and first after the long-term period of observation. This was most probably accomplished by successively restored parathyroid activity from aberrant parathyroid tissue as deducted from data regarding the metabolism of calcium, magnesium and inorganic phosphate. Adaptation, *viz.* normalization of plasma calcium, was brought about by mobilization of skeletal calcium with resulting osteoporosis, as reported in our previous study on the same animals. Intestinal net absorption of calcium showed no significant difference between normocalcemic normophosphatemic intact and hypocalcemic hyperphosphatemic parathyroidectomized animals at the respective level of dietary calcium. Thus, parathyroidectomized animals with persistently reduced plasma calcium showed a normal adaptory increase in intestinal calcium absorption upon chronically restricted calcium intake. In the discussion of the regulation of this adaptory system the possible effects of the plasma concentrations of both calcium and inorganic phosphate upon the intracellular Ca^{++} concentration in the kidney tubules must be considered. Plasma magnesium did not appear to have a primary influence. Intestinal absorption of inorganic phosphate was not dependent upon the presence of the parathyroids but followed that of calcium suggesting a regulation by the action of 1,25-dihydroxycholecalciferol.

Key words Calcium deficiency calcium metabolism metabolism of inorganic phosphate, parathyroidectomy osteoporosis.

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During long-term adaptation to a low calcium intake *i.e.* maintenance of the plasma calcium at a normal level, skeletal calcium is mobilized through the action of the parathyroids (*c.f. Sevastikoglou* & *Larsson* 1972)

with resulting osteoporosis (*c f Larsson* 1969) One-year-old, selectively parathyroidectomized rats cannot adapt themselves to a reduced calcium intake because of inability to mobilize skeletal calcium to the blood in the absence of or at a low level of parathyroid hormone despite an adequate supply of dietary vitamin D (*Larsson & Ahlgren* 1975 a, *Ahlgren & Larsson* 1975 a and b) There is no disturbance of intestinal calcium absorption (*Ahlgren & Larsson* 1975 a) which is regulated by the action of the active metabolite of vitamin D 1,25-dihydroxycholecalciferol (*Holick et al.* 1971 *Myrtle & Norman* 1971) Urinary calcium excretion is reduced because of the reduced plasma calcium level (*Ahlgren & Larsson* 1975 a) Calcium-deficient parathyroidectomized animals show a twicefold increase in intestinal net absorption of calcium (*Ahlgren & Larsson* 1975 a) which suggests either an increased level of circulating 1,25-dihydroxycholecalciferol or as recently suggested by *Favus et al.* (1974) a selective increased accumulation of this metabolite in the intestinal mucosa. Metabolites of vitamin D are also most potent stimulators of bone resorption (*Trummel et al.* 1969 *Raisz et al.* 1972, *Reynolds et al.* 1973) Since calcium-deficient parathyroidectomized animals do not develop osteoporosis and a paradoxical increase in bone mass occurs in parathyroidectomized animals with normal calcium intake and persistently reduced plasma calcium level (*Larsson & Ahlgren* 1975 a *Ahlgren & Larsson* 1975 b) it is suggested that the resorptive action of vitamin D metabolites on bone is present only in concert with parathyroid hormone or is considerably reduced at reduced levels of this hormone (*Ahlgren & Larsson* 1975 b)

The present *long-term* investigation aims at examining the possible progress or reversibility of the *short-term* effects of parathyroidectomy on the metabolism of calcium and inorganic phosphate which we have described previously (*Ahlgren & Larsson* 1975 a) Except for the possibility of aberrant parathyroid tissue which might successively restore parathyroid activity (*c.f Ahlgren & Larsson* 1975 b) adaptation might also be brought about by a possible change in the activity of other mechanisms involved, f.i. vitamin D metabolism.

MATERIAL AND METHODS

Experimental Animals and Housing

Detailed descriptions have been given in our previous investigations (*Larsson & Ahlgren* 1975 a, *Ahlgren & Larsson* 1975 b) which are referred to. Sterile bred male rats of the Sprague-Dawley strain* were used in the present long-term series comprising one observation period of 6 months. All animals were stated to be 12 to 12½ months old at the start of the experiment and their initial body weight were 515–645 grams.

Experimental Diets

The low calcium diet of General Biochemical Inc., Chagrin Falls, Ohio, was used as a basic ration (*c.f Larsson 1969*) The diet** contained 0.04 per cent Ca and 0.70 per cent P with a Ca P ratio of 1:17.5 and was supplied with vitamin D_3 600 I.U per 100 g diet. The amount of Mg in the diet was 0.20 per cent.

The normal calcium diet consisted of the low calcium test diet supplemented with $CaCO_3$, this diet thus containing a total amount of 1.2 per cent Ca and 0.70 per cent P with a Ca P ratio of 1-0.55

Diets*** and deionized water were given *ad libitum*.

Parathyroidectomy (PTE)

This was performed as described previously (*c f Larsson & Ahlgren* 1975) Only animals which showed a postoperative fall in the plasma calcium level to below 4 1 mEq./l were accepted as PTE animals (*c.f Johansson & Segerström* 1972) The following groups of animals were obtained for the present series depending upon treatment and plasma calcium level

Experimental animals. PTE animals were divided into the following groups of the long term series

PTE — Ca twenty-one PTE rats supplied the low calcium diet showing a consistently low plasma calcium level.

PTE + Ca six PTE rats supplied the normal calcium diet showing a consistently low plasma calcium level.

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* Obtained from Mollegaard Hansens Avlslaboratorier A/S, Ejby Denmark.

** Here and in the following text the figures calculated on a dry weight basis are given.

*** Obtained from Ewos Co., Södertälje Sweden.

PTE_N — Ca three PTE rats given the low calcium diet showing a normalization of the plasma calcium level after an initial reduction.

PTE_N + Ca eight PTE rats supplied the normal calcium diet having normalized their plasma calcium level after an initial reduction.

Intact animals were randomly divided into the following groups

— Ca seven animals supplied the low calcium diet.

+ Ca; eight animals given the normal calcium diet thus constituting the control group.

Isotope Administration

Seventy-two hours before sacrifice, each animal received 20 microcuries of ^{45}Ca in 1.0 ml physiological saline solution from a single batch of $^{45}CaCl_2$ for the whole series. The animals were thereafter kept isolated in metabolic cages until sacrifice. The isotope solution was administered by stomach tube under light ether anaesthesia as described previously (*Ahlgren* & *Larsson* 1975 a)

Plasma Sampling

From each animal approximately 0.5–1.0 ml of blood was taken by cardiac puncture under light ether anaesthesia 2 days, 4 6 and 14 weeks after the start of the experiment and 4 24 48 and 72 hours before sacrifice of the animal. Finally blood was collected at the time of sacrifice which was performed by bleeding the animal to death from the femoral artery under ether anaesthesia The collected blood was centrifuged at 3,000 r p.m. for 15 minutes and the supernatant plasma was pipetted into acid washed test tubes and kept at 20° C pending subsequent analyses.

The following determinations were performed as described previously (*Ahlgren* & *Larsson* 1975 a)

Plasma Calcium and Magnesium
Inorganic Phosphate of Plasma
Urinary Calcium Magnesium and Inorganic Phosphate
Faecal Calcium Magnesium and Inorganic Phosphate
^{45}Ca Activity of Plasma, Urine Faeces and Tibia
Calcium Accretion Rate by Bone

Calculation of intestinal net absorption of calcium and inorganic phosphate was performed as described by *Nordin* (1963)

Calcium chloride in aqueous solution 2–10 mCi/mg Ca, CES. 2, The Radiochemical Centre Amersham, England.

Statistical Treatment of Data

Data applying to the different groups were compared in pairs and subjected to the Student's t test. The hypothesis of no difference between groups was tested with a 5 per cent significance level.

RESULTS

Mortality

This has been described in our previous study (*Ahlgren* & *Larsson* 1975 b) which is referred to

Plasma Calcium Faecal and Urinary Calcium

For intact animals, plasma calcium showed no significant difference between rats fed the low calcium diet and those supplied the calcium supplemented diet (Fig 1)

Among the PTE animals belonging to the long term series (*cf* *Ahlgren* & *Larsson*

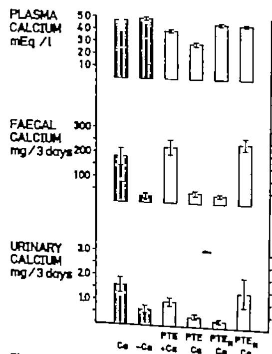


Fig 1 Plasma calcium, faecal and urinary calcium of the normal control group and the various experimental groups (+ Ca = normal dietary calcium — Ca = low dietary calcium PTE = parathyroidectomy PTE_N = PTE animals showing a normalized plasma calcium level) The bars represent the standard error of the mean.

1975 b) another 40 per cent of those with a normal calcium intake showed a normalization of their plasma calcium between the 8th and 27th week of observation ($PTE_N + Ca$), and almost 10 per cent of those with a low calcium intake ($PTE_N - Ca$)

Among PTE animals with persistently reduced plasma calcium level, those with a normal calcium intake showed a mean value that was 18.8 per cent lower than normal and those with a low calcium intake 39 6 per cent lower the differences being highly significant.

Faecal calcium was significantly lower ($0.001 < p < 0.005$) in animals with low calcium intake than in those with normal calcium intake, as was expected. Within the groups of rats supplied the low and normal calcium diet, respectively no significant difference was obtained.

Urinary calcium was significantly lower ($0.01 < p < 0.02$) in intact animals with low calcium intake compared with those with normal calcium intake. PTE animals with reduced plasma calcium showed also lower ($0.01 < p < 0.02$) values at a low level of dietary calcium than at a normal level. However in these groups of rats urinary calcium appeared to reflect the plasma calcium level primarily At respective level of dietary calcium, the PTE animals showed a tendency at lower values than those of the intact animals but the differences were not significant. For PTE animals with normalized plasma calcium, values were obtained which were not significantly different from those of the intact animals neither at a normal nor at the low level of calcium intake.

The ⁴⁵Ca-Activity of Plasma, Faeces Urine and Bone

The ⁴⁵Ca-activity of plasma was expressed in per cent of administered dose per mg plasma calcium (Fig. 2) For intact animals, higher ($p < 0.001$) values wer obtained at the low calcium intake than at the normal. PTE animals with reduced plasma calcium showed also higher ($p < 0.001$) values at the low compared with at the normal calcium intake.

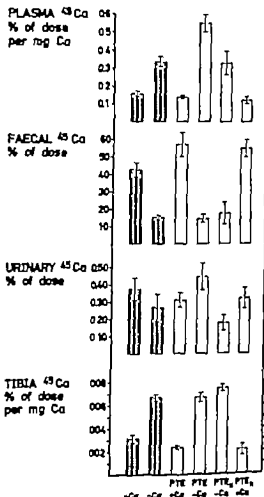


Fig. 2 The ⁴⁵Ca-activity of plasma, faeces, urine and bone of the same groups of animals as in Fig. 1. The isotope was administered by stomach tube 72 hours before sacrifice. Note the higher ⁴⁵Ca-activity of plasma and bone with lower ⁴⁵Ca-activity of faeces in both calcium-deficient intact and PTE animals than in the intact control animals.

PTE animals with reduced plasma calcium showed at a normal calcium intake similar values and at a low calcium intake higher ($0.02 < p < 0.025$) values compared with those of the intact animals. PTE animals with normalized plasma calcium showed values which were similar to those of the intact animals both at the normal and at the low level of dietary calcium, respectively

The faecal ⁴⁵Ca-activity expressed as per cent of administered dose showed significantly lower values in all groups of rats fed the

low calcium diet compared with those supplied the calcium supplemented diet (Fig. 2). At a normal calcium intake, PTE animals showed almost significantly ($0.05<p<0.10$) higher values than those of the intact animals while at a low intake of calcium no differences were found.

The urinary ⁴⁵Ca-activity expressed in per cent of dose, showed no significant differences within the experimental series (Fig. 2).

The ⁴⁵Ca-activity of the tibia, expressed in per cent of dose per mg calcium of the bone (see *Larsson* 1969) showed no significant differences between intact and PTE animals neither at a normal nor at a low calcium intake (Fig. 2). Both for intact and PTE animals more than twice as high values were obtained in those fed the low calcium diet as in those supplied the calcium supplemented diet. PTE animals with normalized plasma calcium level showed no significant difference from those with consistently reduced plasma calcium at respective level of dietary calcium.

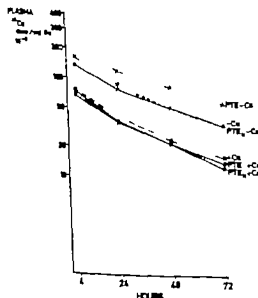


Fig. 3. The plasma ⁴⁵Ca-activity at different periods after the administration of the isotope by stomach tube. Note the higher activity in all calcium-deficient animals compared with intact animals, and especially in the group of PTE −Ca animals. This difference was found also when differences in body weight were taken into consideration.

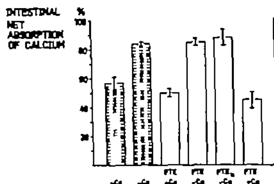


Fig. 4. Intestinal net absorption of calcium. At a low intake of calcium, significantly higher values were obtained for both intact and PTE animals compared with those obtained for both the intact controls and the PTE animals fed the normal calcium diet as well.

The Plasma ⁴⁵Ca Activity at Different Periods after the ⁴⁵Ca Administration

The ⁴⁵Ca-activity of plasma, expressed in per cent of dose per mg calcium of the plasma, was considerably higher in all groups of animals supplied the low calcium diet than in the normal control group (Fig. 3). At the low level of calcium intake, both the group of intact animals and that of the PTE animals with normalized plasma calcium showed an increase by 110 per cent while in the PTE animals with reduced plasma calcium the increase was 240 per cent. These differences were found also when differences in body weight were taken into consideration. At a normal calcium intake, PTE animals showed a tendency at reduced values but the difference was not significant.

Intestinal Net Absorption of Calcium

In the intact animals, significantly higher ($p<0.001$) values were obtained in the group of rats fed the low calcium diet than in those supplied the calcium supplemented diet, the difference being almost 50 per cent (Fig. 4). The same observation was made also in PTE animals with reduced plasma calcium, the difference here being more than 70 per cent. Intact and PTE animals showed no significant difference neither at a normal nor at a low level of dietary calcium. Likewise, there

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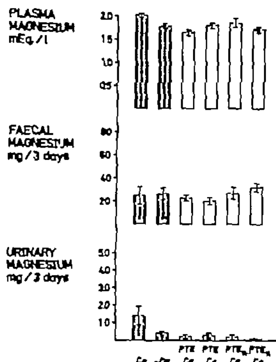


Fig. 5. Plasma magnesium, faecal and urinary magnesium. Note the lower values for urinary magnesium obtained for all experimental groups compared with those of the control group.

was no significant difference between PTE animals with normalized and those with consistently reduced plasma calcium.

Plasma Magnesium, Faecal and Urinary Magnesium

Plasma magnesium was lower ($0.001<p<0.005$) in calcium-deficient intact animals than in the normal controls (Fig. 5). For the various groups of PTE animals, similar values were obtained. At a normal calcium intake lower ($p<0.001$) values were found in the two groups of PTE rats with reduced and normalized plasma calcium in comparison with those of the intact controls. At the low level of calcium intake no such difference was found.

Faecal magnesium showed no significant difference from normal in any of the experimental groups (Fig. 5). For PTE animals with normal calcium intake and normalized plasma calcium higher ($p<0.05$) values were obtained compared with those of the corresponding group with consistently reduced plasma calcium and also in comparison with those of the calcium-deficient PTE rats with reduced plasma calcium ($0.025<p<0.05$).

Urinary magnesium showed lower values in all experimental groups than in the normal control group (Fig. 5), the differences being significant ($0.025<p<0.05$) for the groups of PTE animals with low calcium intake having reduced plasma calcium and that of the PTE animals with normal calcium intake and normalized plasma calcium. For the latter group very low values were obtained which were lower ($0.001<p<0.005$) than those of the calcium-deficient intact animals.

Plasma and Urinary Calcium and Inorganic Phosphate

In plasma, the contents of inorganic phosphate showed an inverse relationship to those of calcium (Fig. 6). In comparison to normal, significantly higher values for inorganic phosphate were obtained for the two groups of hypocalcemic animals, namely those of PTE rats with normal ($0.01<p<0.02$) and low calcium intake ($p<0.001$). The latter group showed higher ($p<0.001$) values than the former. For the two groups of PTE rats with normalized plasma calcium, values were obtained which were not different from normal but higher ($0.025<p<0.05$) than those of the calcium-deficient intact animals.

In urine, higher ($p<0.001$) values for inorganic phosphate were obtained for the group of calcium-deficient intact animals compared with those of the normal controls. At a normal calcium intake PTE animals showed a tendency at higher values than normal but the difference was not significant. At a low calcium intake the two groups of PTE animals showed highly increased ($p<0.001$) values which were similar to those of the calcium-deficient intact animals. The group of PTE rats with normal calcium intake and normalized plasma calcium showed no significant difference from normal.

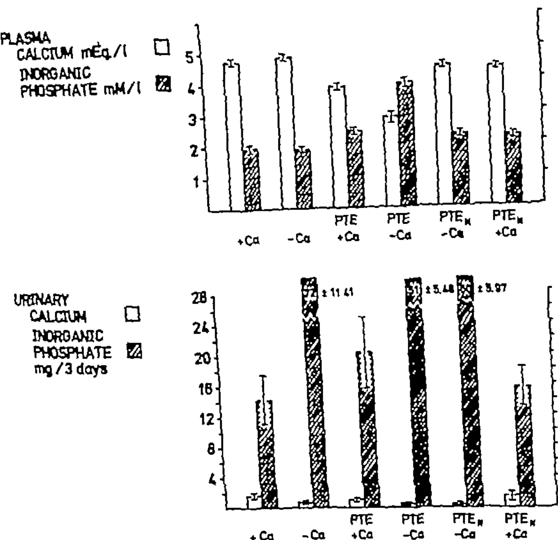


Fig. 6. Plasma and urinary levels of calcium and inorganic phosphate. The contents of inorganic phosphate in plasma showed an inverse relationship to those of calcium. Note the highly increased urinary phosphate in the calcium-deficient intact animals compared to normal. The increased urinary phosphate in the calcium-deficient PTE animals was certainly due to increased clearance of the markedly elevated plasma phosphate in these animals.

Inorganic Phosphate of Plasma, Faeces and Urine

In comparison with the normal control group, the group of calcium-deficient intact animals showed similar contents in plasma, increased in urine and reduced ($0.025 < p < 0.05$) in faeces (Fig. 7). PTE animals with normal calcium intake and reduced plasma calcium showed increased contents in plasma and normal contents in urine and faeces. The corresponding group with low calcium intake showed highly increased contents in plasma and urine and reduced ($p < 0.001$) contents in faeces. For the group of calcium-deficient PTE rats with normalized plasma calcium normal contents were obtained in plasma, highly increased in urine and reduced ($p < 0.001$) in faeces. The group of PTE animals with normal calcium intake and normalized plasma calcium showed no significant differences from normal.

While faecal inorganic phosphate was sig-

nificantly reduced in all groups of rats kept on the low level of calcium intake, it showed no significant difference between intact and PTE animals at the respective level of dietary calcium.

Intestinal Net Absorption of Calcium and Inorganic Phosphate

As shown in Fig 8, there was a strikingly positive relationship between the two parameters. As for calcium, higher ($0.001 < p < 0.005$) values were obtained for the net absorption of inorganic phosphate in the calcium-deficient intact animals compared with those of the intact controls. Net absorption of both calcium and inorganic phosphate was significantly higher in all groups of rats with low calcium intake than in those fed the calcium supplemented diet. At the respective level of dietary calcium intake, there were no significant differences between intact and PTE animals.

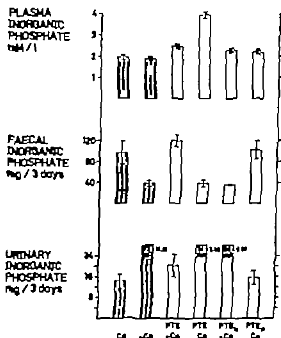


Fig 7 Inorganic phosphate of plasma, faeces and urine. The calcium-deficient intact animals showed increased contents in urine and reduced in faeces. For PTE animals with low calcium intake highly increased contents were obtained in plasma and urine and reduced in faeces.

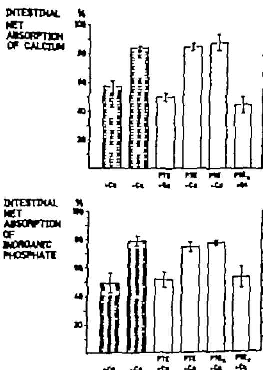


Fig 8 Intestinal net absorption of calcium and inorganic phosphate showed a strong positive correlation to each other

DISCUSSION

At a normal dietary intake of calcium, phosphorus and vitamin D 17 per cent of one-year-old rats with a verified parathyroidectomy normalized their plasma calcium within 8 weeks (*Larsson & Ahlgren* 1975 a) and another 40 per cent between the 8th and 27th week of observation (*Ahlgren & Larsson* 1975 b) At a low calcium intake, a normalization occurred in only 10 per cent and first after the long term period of observation (*Ahlgren & Larsson* 1975 b) Normalization was brought about by mobilization of skeletal calcium through increased bone resorption with resulting osteoporosis (*Ahlgren & Larsson* 1975 b) As discussed in our previous study (*Ahlgren & Larsson* 1975 b) this was most probably accomplished by successively restored parathyroid activity from aberrant parathyroid tissue. The results of the present investigation give further strong support for this interpretation because these two groups of animals were comparable to their intact

controls as regards the metabolism of calcium, magnesium and inorganic phosphate indicat ing a higher parathyroid activity at the low than at the normal level of calcium intake (*Severitkoglou & Larsson* 1972 *Ahlgren & Larsson* 1975 a) This was reflected in the highly increased urinary excretion of inor ganic phosphate in the calcium-deficient animals.

The remaining two groups of parathyroid-ectomized animals showed a persistently reduced plasma calcium level with no tendency at a normalization even after more than 6 months of observation. As shown in our previous study (*Ahlgren & Larsson* 1975 b) the degree of plasma calcium reduction was related to the level of dietary calcium intake and to a reduced ability to mobilize skeletal calcium to the blood. The data of the present investigation indicate that this was the main cause of the inability of adaptation similar to the situation after a short-term period of observation (*Ahlgren & Larsson* 1975 a) Intestinal net absorption of calcium was significantly increased at a low dietary intake of calcium in both intact and parathyroid ectomized animals. While after short term (8 weeks) observation parathyroidectomized animals showed a tendency at increased absorption on the normal level of dietary calcium and a twicefold increase on the low level, in this long-term investigation (6 months) parathyroidectomized animals showed no significant difference from normal at respective level of calcium intake. This difference is best explained by the fact that the animals of the long-term series of natural reasons were older than those of the short-term series at the end of the experiment. The intact controls of the present series had a higher net absorption of calcium than those of the short term series but the increase in the calcium-deficient parathyroidectomized animals reached the same level in the two series. The findings made in the intact animals are consistent with the report by *Hironaka* et al. (1960) that aged rats (22 to 32 months compared to 10 to 12 months) are subject to a greater loss of calcium from the body but that they are able to maintain calcium equilibrium by increasing the uptake of dietary calcium from the intestine. In addition, we found a reduction of both faecal and urinary calcium with age. There was also quite a slight reduction of the spongious bone but not of the cortical bone with age (*Larsson & Ahlgren* 1975 a, *Ahlgren & Larsson* 1975 b)

The results of the present investigation demonstrate that the adaptory increase in intestinal net absorption of calcium to chronic dietary calcium restriction can definitely occur in adult animals with a verified parathyroidectomy This confirms our short term observations (*Ahlgren & Larsson* 1975 a) Similar results have recently been obtained in a short term study of thyro-parathyroidectomized rats supplemented with L-thyroxine and fed a low calcium diet for 3 weeks (*Favus et al* 1974)

As shown by *Nicolaysen et al.* (1953) vitamin D is essential for adaptation to occur It is now well established that the most active metabolite of vitamin D is 1,25-dihydroxycholecalciferol. The acute experiments of *Boyle et al.* (1971) on the effect of dietary calcium on the production of vitamin D metabolites suggested that 1,25-dihydroxycholecalciferol is responsible for the adaptation of calcium absorption to low dietary concentrations of calcium. At present, there is considerable speculation as to the mechanism controlling this conversion. In thyroparathyroidectomized young rats and in parathyroid ectomized chicks reduced concentrations of 1,25-dihydroxycholecalciferol have been reported both in plasma and in intestine (*Garabedian et al.* 1972, *Fraser & Kodicek* 1973) and in rats an increased production has been found after treatment with parathyroid extract (*Garabedian et al.* 1972) Parathyroid hormone has therefore been suggested to act as a tropic hormone for 1,25-dihydroxycholecalciferol, which in turn is responsible for intestinal calcium absorption and bone calcium mobilization (*cf. Omdahl & DeLuca* 1973, *Arnoli & Haddad* 1973) Results of *in vitro* studies using isolated renal tubules and *in vivo* studies suggest a stimulatory effect of para-

thyroid hormone on the 1-hydroxylation system necessary for the conversion of vitamin D into 1,25-dihydroxycholecalciferol (*Shain* 1972, *Rasmussen et al* 1972, *Fraser & Kodicek* 1972) Acutely increased conversion has recently been reported, however in parathyroidectomized young rats (*Larkins et al* 1973) and a suppressive effect of parathyroid extract (*Galante et al.* 1972) *Ferus et al* (1974) found reduced levels of 1,25-dihydroxycholecalciferol in the plasma but a selective accumulation in the intestinal mucosa of thyroparathyroidectomized young rats supplemented with L-thyroxine and fed a low calcium diet. Studies on acute effects of parathyroidectomy and removal of the ultimobranchial glands in chicks have now led *Galante et al.* (1973) to the conclusion that "parathyroid hormone is not an essential hormone for the production of 1,25-dihydroxycholecalciferol" Thus, opinions differ on the role of parathyroid hormone in this adaptation phenomenon. Our calcium metabolic data indicate no direct regulatory influence of the parathyroid glands upon the metabolism of vitamin D in the adult rat neither after short term nor after long term periods of observation. Our current studies of the vitamin D metabolism under these circumstances give firm support for this conclusion. Production of 1,25-dihydroxycholecalciferol occurs both in intact and in selectively parathyroidectomized animals (*Larsson et al.* 1975)

Most recently changes in intracellular calcium of the renal tubular cell have been considered to be responsible for the effects of parathyroid hormone on the production of 1,25-dihydroxycholecalciferol (*Colston et al.* 1973 *Galante et al.* 1974) The 1 hydroxylase activity of chick kidney homogenates responds instantaneously and inversely to intracellular changes of Ca concentration, the formation of 1,25-dihydroxycholecalciferol being inhibited by increasing concentration of Ca^{2+} in the medium. Possibly this mechanism is responsible for the adaptory increase in intestinal calcium absorption upon reduced calcium intake In the interpretation of our results it becomes evident that intracellular Ca^{2+} concentration is not reflected by the plasma calcium concentration solely but also by that of inorganic phosphate among others. Both at the normal and at the low level of dietary calcium there was no significant difference in intestinal net absorption of calcium between the intact normocalcemic, normophosphatemic and the parathyroidectomized hypocalcemic, hyperphosphatemic animals. The difference in calcium absorption found between the parathyroidectomized animals with a normal and a low calcium intake would then be explained by the circumstance that in the former the mean reduction in plasma calcium by 19 per cent would not result in reduced intracellular Ca^{2+} because of the concomitant increase in inorganic phosphate by 27 per cent. In the latter the mean reduction in plasma calcium by 40 per cent was too profound that intracellular Ca^{2+} would be maintained at a normal level by the increase in inorganic phosphate that amounted to 50 per cent. Therefore calcium absorption was increased in the latter despite the absence of the parathyroids. An increased level of circulating 1,25-dihydroxycholecalciferol in this group of animals is also suggested from the finding of maintained bone resorption in the absence of the parathyroids, while in the former group an increase in bone mass occurred (*Ahlgren & Larsson* 1975 b) In the calcium-deficient, intact animals showing normal plasma concentrations of calcium and inorganic phosphate the increased calcium absorption would also be explained by lowered intracellular Ca^{2+} in the kidney tubules.

In conclusion we found a normal adaptory increase in intestinal calcium absorption in parathyroidectomized adult animals in a chronic state of restricted calcium intake These findings suggest indirectly that the metabolism of vitamin D is not regulated by the parathyroids. Further in the regulation of this adaptory system the possible effects of the plasma concentrations of both calcium and inorganic phosphate upon intracellular Ca concentration in the kidney tubules must be considered. Plasma magnesium and magnesium metabolism did not appear to have a

primary influence under these circumstances.

In the present investigation, a strong positive correlation was found between the intestinal net absorption of calcium and inorganic phosphate. The absorption of inorganic phosphate was not dependent upon the presence of the parathyroids but seemed to be dependent upon the level of dietary calcium intake. This would result in a further increase in the concentration of plasma inorganic phosphate in the hypocalcemic parathyroidectomized animals and further increased renal filtered load and urinary phosphate excretion. Under the present circumstances, the intestinal absorption of phosphate like that of calcium might be regulated by the action of 1,25-dihydroxycholecalciferol. It might also be secondary to the different levels of dietary calcium intake used in the present investigation as reported by *Clark* & *Rivera-Cordero* (1973)

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FOCAL NODULAR HYPERPLASIA OF THE LIVER, BENIGN HEPATOMAS, ORAL CONTRACEPTIVES AND OTHER DRUGS AFFECTING THE LIVER

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Grabowski, M., Stenram, U. & Bergqvist, A. Focal nodular hyperplasia of the liver, benign hepatomas, oral contraceptives and other drugs affecting the liver. Acta path. microbiol. scand. Sect. A, 83: 615–622, 1975.

Due to the claim of an association between focal nodular hyperplasia of the liver, benign hepatomas and oral contraceptives, the files in the departments of pathology at the university hospitals in Lund and Malmö were examined for these two diagnoses made since 1945 and 1957 respectively. 26 cases of focal nodular hyperplasia of the liver and 7 benign hepatomas were found, 18 and 2, respectively, in women. Since 1963, the year before oral contraceptives were introduced in Sweden, focal nodular hyperplasia has been diagnosed in 8 women in the reproductive period of life; 4 of these had taken oral contraceptives. The 4 diagnoses were established in 1972–1974. At most, 25 per cent of Swedish females between the ages of 15 and 44 years have been on oral contraceptives. The Swedish series of 28 patients with focal nodular hyperplasia comprised 3 epileptics and 3 diabetics. At least two of the epileptics had been treated with barbiturates and/or hydantoins. The prevalence of drug-treated epilepsy in Sweden is 0.4–0.5 per cent, and of diabetes about 2 per cent. The possible aetiological role of drugs provoking an increase of the smooth-surfaced endoplasmic reticulum of the liver and proliferation of vascular fibrous tissue in a part of the liver which previously may have been damaged by vascular disturbances or trauma, is considered. No relationship between benign hepatomas and drugs was found. The observations support the notion that oral contraceptives may be of aetiological importance in the development of focal nodular hyperplasia of the liver although the material is too small for epidemiological and statistical analysis.

Key words: Liver; focal nodular hyperplasia; hepatomas; oral contraceptives.

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Benign hepatomas are tumours consisting of liver parenchymal cells but lacking bile ducts. The condition is probably often premalignant. Focal nodular hyperplasia of the liver is a circumscribed tumour-like condition in which areas of liver parenchymal cells are separated by fibrous tracts containing varying numbers of bile ducts, and is thus histologically very similar to cirrhosis. A central fibrous scar like area is as a rule visible macroscopically (cf. Fig. 1). Dilated, thin-



Fig 1 The central fibrous scar-like area, characteristic of focal nodular hyperplasia of the liver Bile ducts are seen as dark tubules in slender fibrous tracts. Case 10 Htx-eosin. × 25

walled vessels are often present, which explains the tendency to haemoperitoneum, sometimes with a fatal outcome.

Since October 1973 at least 21 cases of hepatic adenoma or focal nodular hyperplasia of the liver in women on oral contraceptives have been described (*Baum et al.* 1973 *Mays et al.* 1974 *Berg et al.* 1974 *Editor Brit Med J* 1974 *Vosnides et al.* 1974) According to our knowledge, only two cases without relation to oral contraceptives, one in each sex, have been described during this time (*Berg et al.* 1974 *O Sullivan & Wilding* 1974) As *Mays et al* (1974) point out, all cases of which detailed histological descriptions are available represent focal nodular hyperplasia as defined above. Some reports contain very few or no histological data and permit no evaluation of the established diagnosis. Thus the varying histological criteria on which benign hepatoma is diagnosed seem to be the reason why the diagnoses presented differ

Focal nodular hyperplasia was known before the era of oral contraceptives (*Benz & Baggenstoss* 1953) and it was found also in children (*Whelan et al* 1973) We have recently seen a few cases of this entity Against the aforementioned background it was considered of interest, on the basis of our files, to review the cases of benign hepatomas and focal nodular hyperplasia of the liver

MATERIAL

The biopsy and autopsy files in the departments of pathology of the university hospitals in Malmö and Lund were searched for benign hepatomas and focal nodular hyperplasia of the liver—in Lund, biopsies dating back to 1945 and autopsies dating back to 1952, in Malmö, biopsies and autopsies dating back to 1957 and hence forward to November 15 1974 Until 1974 the number of biopsies obtained in Lund has increased from 8,000 in 1945 to 25 000, and the number of autopsies from 500 in 1952 to 1 100 a year In Malmö, the number of biopsies has increased from 7,500 in 1957 to 15,000 and the number of autopsies from 940 to 1,900 a year

Malmö is the third largest city in Sweden, by now with about 250,000 inhabitants. There is only one major hospital where every biopsy from Malmö and a few from other areas are examined. About 70 per cent of individuals who die in Malmö are autopsied at the department of pathology Patients 19 and 20 though examined in Malmö, did not reside in that city

The university hospital in Lund is a regional hospital at present accommodating about 1,200,000 people and a local hospital accomodating about 160,000 people.

The slides from cases diagnosed as benign hepatoma and focal nodular hyperplasia of the liver were re-examined using the criteria summarized in the introduction. The patient records were scrutinized. Females in the reproductive period of life were interviewed about their possible intake of oral contraceptives. The pertinent cases together with brief clinical data are recorded in the Tables.

RESULTS

Seven cases of *benign hepatomas* were diagnosed in the period 1960–1974 Five of these (4 in males and 1 in a female) were accidental findings at autopsy of patients in the age range 54–88 years. None was larger than 5 cm. The causes of death were malignant glioma bronchial asthma—cortisone treated—with gastric bleeding myocardial infarct encephalomalacia bronchopneumonia.

The tumours gave rise to *symptoms* in 2 patients. A 37-year-old insulin-treated dia

betic women was found to have an oppression in the abdomen, and a benign hepatoma weighing 1.5 kg was excised. The patient died 6 years later without recurrence, in diabetic nephropathy. A 32-year-old male with abdominal pains persisting throughout 3 years and associated with fever and elevated ESR had a 12 cm large hepatoma with some cellular polymorphism. Only biopsy could be performed. There was no progress of the tumour during the following 6 months. The patient had not taken drugs regularly.

Twenty-six cases of *focal nodular hyperplasia of the liver* were found (Tables 1 and 2) including one previously published (*Aronsen et al.* 1968). Eleven (4 males, 7 females) were accidental findings at autopsy. Twelve (11 females, 1 male) were accidental findings at laparotomy, 7 of these at cholecystectomy.

Only 2 patients had symptoms that might be ascribed to the liver lesions, cases 5 and 7.

It has come to our knowledge that still another case of either benign hepatoma or focal nodular hyperplasia of the liver has been observed in the university clinic in Lund. The diagnosis was established only by angiography, a method that does not discriminate between these two conditions. The patient was a 17-year-old female with heterozygous α_1-antitrypsin deficiency. She had been a diabetic for 5 years, and she was not on oral contraceptives (Zurbriggen and Tylén, 1975). None of the cases discussed in the present report displayed the cytoplasmic globules characteristic of α_1-antitrypsin deficiency.

DISCUSSION

In Sweden, the percentage of the female population in the agegroup 15 to 44 years who had oral contraceptives was in 1964 1 per cent, in 1965 7 per cent, in 1966 10 per cent, in 1967 16 per cent, in 1968 20 per cent, in 1969 25 per cent, in 1970 24 per cent, in 1971 22 per cent, and in 1973 19 per cent (*Westerholm* 1974). In the present study a series of 8 cases of focal nodular hyperplasia of the liver which were diagnosed 1964–1974 in women between 15 and 50 years, included 4 who had taken oral contraceptives. All 4 diagnoses established in 1972–1974 were in patients on oral contraceptives (cases 8–10 and 26). These cases and those reported in the literature suggest that the use of oral contraceptives for some years may imply a risk of a development of focal nodular hyperplasia of the liver. The annual rate in Sweden may be, roughly, 1 per 10 000 women at risk after 5 to 10 years on oral contraceptives.

In case no. 6, epilepsy had persisted for 30 years, and, in recent years—exactly how long is not known—the patient had been treated with hydantoins, a drug introduced in Sweden in 1940; previously treatment may have included barbiturates. Case no. 11 was also an epileptic, with a clinical history of 41 years, treated in a similar way.

Von Sydow (1968), Sweden, reported 2 patients in whom multiple lesions of focal nodular hyperplasia were manifest, one patient was a 36-year-old woman in whom diabetes had persisted for 10 years, the other was a 38-year-old woman with epilepsy of unknown duration. Thus, in a series of 28 Swedish patients with focal nodular hyperplasia observed by us and von Sydow, three have been epileptics. In Sweden, the prevalence of epilepsy in patients on continuous drug therapy is estimated to 0.4–0.5 per cent.

In addition, patients nos. 15, 21, 23, 24 and 25 had for some time been on barbiturates, patient no. 21 also on diazepam.

Two of our patients with focal nodular hyperplasia were diabetics. One of these had also epilepsy and has been mentioned above. Also one of the patients described by von Sydow was a diabetic. That makes 3 diabetics in a series of 28 Swedish cases of focal nodular hyperplasia. In Sweden the prevalence of diabetes is about 2 per cent.

It applies to most papers on focal nodular hyperplasia that nothing is said about concomitant diseases and drugs. *O'Sullivan & Wilding* (1974) published a report including a man in whom an adrenal carcinoma developed. It was not stated whether it arose



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Case no.	Age at diagnosis (yr)	Sex	Year of diagnosis	Presenting symptoms
1	42	F	1960	Accidental finding at cholecystectomy
2	18	F	1963	Accidental finding at autopsy
3	43	F	1964	Accidental finding at cholecystectomy
4	25	F	1965	Accidental finding at laparotomy motivated by extra uterine pregnancy
5	27	M	1966	Epigastric pain related to food intake. Rise of bilirubin (3.5 mg per cent) Other liver tests normal. Melaena.
6	56	M	1971	Accidental finding at autopsy
7	6	M	1972	Mother noticed oppression in abdomen.
8	24	F	1972	Accidental finding at cholecystectomy
9	25	F	1973	Accidental finding at caesarean section.
10	47	F	1973	Accidental finding at myoma uteri operatio

from the cortex or medulla or it was hormone producing

Of course, our few data do not give any statistical proof of the relationship between focal nodular hyperplasia of the liver and drugs. The fact that this lesion is a common finding in patients on oral contraceptives and/or antiepileptics and in those with endocrine diseases, however is worth mentioning

Since this report was completed, a paper has been published according to which a malignant hepatic tumour a hepatoblastoma, developed in a women on oral contraceptives (*Meyer et al.* 1974) it should be stressed, however that it is the only case of this type so far published.

Some of the drugs dealt with in the present investigation may affect two different components of the liver the parenchymal cells, and the connective tissue and its vessels.

As regards the parenchymal cells, the smooth-surfaced endoplasmic reticulum of the cytoplasm is increased in icteric women as well as in a few non-icteric women on oral contraceptives (*Larsson-Cohn & Stenram* 1965 *Larsson Cohn & Stenram* 1967 *Pihl et al.* 1968 *Perez et al.* 1969) Progesterone, given to patient no. 13 increases the smooth-surfaced endoplasmic reticulum of the liver in rats and has several effects on the human liver (*Adlercreutz & Tenhunen* 1970) Diazepam, taken by patient 21 is also meta-

Size of lesion and management	Drugs and concurrent diseases	Follow-up
3 cm. Excision of tumour	Previously healthy No drugs known.	Postoperatively well.
3 cm.	Previously healthy No drugs regularly	Cause of death: acute meningococcal sepsis and anuria.
3 cm. Excision of tumour.	Psychiatric disturbances. During 1964 treated with chlordiazepoxide, glutethimide, nialamide, oxyphencyclimine, meprobamate. No oral contraceptives.	Alive.
12 × 4 × 4 cm. Resection of left and quadrate lobes.	Previously healthy No drugs regularly No oral contraceptives.	Alive.
3 cm. Excision of tumour of right lobe.	Poliomyelitis in childhood. No sequelae. No drugs regularly	Since 1966 bilirubin rise (1.5 mg per cent)
1 × 2 cm.	Since 1930 epilepsy treated with diphenylhydantoin. Since 1960 diabetes treated with phenformin and insulin.	Cause of death. perforating gastric ulcer
3 cm. Excision of tumour of left lobe.	Healthy No drugs regularly	Postoperatively well
cm. Excision of tumour.	Lyndiol mite® (lynestrenole 2.5 mg, mestranole 0.075 mg) 5 yrs.	Alive.
cm. Excision of tumour of right lobe.	Lyndiol mite® (lynestrenole 2.5 mg, mestranole 0.075 mg) 5 yrs, ended 1970.	Alive.
× 7 × 5 cm. Excision of tumour of right lobe.	Norethisterone, methyloestrenolone several years before 1962. Anovlar mite® (norethisterone 3 mg, ethinylestradiol 0.05 mg) since 1962 and other unknown drugs for psychiatric disturbances.	Committed suicide. No autopsy

bolized in the liver and, in therapeutic doses, it increases the smooth-surfaced endoplasmic reticulum in man (*Jezequel et al.* 1974) and the same appears to hold for hydantoins and barbiturates which may even produce osteomalacia in epileptics, owing to the increased metabolism of vitamin D (*Dent et al.* 1970, *Richens & Rowe* 1970).

Gingival hyperplasia is a well known side effect of hydantoins and occasionally it has been observed in women on oral contraceptives (*Kaufman & Gee* 1969). The related pregnancy gingivitis (pregnancy tumour, epulis gravidarum) is characterized by dilated thin-walled vessels (*Tillilä* 1962), very similar to those seen in connective tissue stroma in cases of focal nodular hyperplasia of the liver. Pregnancy gingivitis resembles granuloma pyogenicum which is said to be most common in women, notably in pregnant women (*Tillilä* 1962). In cirrhotic patients spiders have been considered to be due to increased activity of estrogens, decreased activity of androgens, or both.

Oral contraceptives and hydantoins thus affect the liver parenchymal cells as well as the fibrous vascular tissue, which may explain their role in the aetiology of focal nodular hyperplasia of the liver.

Apart from the recent papers referred to in the introduction, claiming a relationship between oral contraceptives and focal nodular

TABLE 2. *Focal Nodular*

Case no.	Age at diagnosis (yr)	Sex	Year of diagnosis	Presenting symptoms
11	56	M	1959	Accidental finding at autopsy
12	57	M	1961	Accidental finding at autopsy
13	76	F	1962	Accidental finding at autopsy
14	35	F	1962	Accidental finding at autopsy
15	68	F	1963	Accidental finding at autopsy
16	65	F	1965	Accidental finding at autopsy
17	37	F	1965	Accidental finding at cholecystectomy
18	46	F	1965	Accidental finding at cholecystectomy
19	65	M	1966	Accidental finding at explorative laparotomy for appendicitis
20	23	M	1967	Accidental finding t coeliac angiography because of abdominal trauma.
21	60	F	1968	Accidental finding at cholecystectomy
22	59	F	1969	Accidental finding at cholecystectomy
23	91	F	1970	Accidental finding at autopsy
24	60	M	1970	Accidental finding at autopsy
25	71	F	1973	Accidental finding at autopsy
26	28	F	1974	Accidental finding at cholecystectomy

hyperplasia of the liver and benign hepatomas, focal nodular hyperplasia of the liver has generally been considered to arise on the basis of vascular anomalies, perhaps due to trauma (*Aronsen et al* 1968) or malformation (*Whelan et al.* 1973)

Whether oral contraceptives and hydantoins might provoke a reaction in a normal liver or in a liver with small areas of vascular disturbances or damaged in some way by previous trauma is not known

No relationship between benign hepatomas and drugs was found.

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NEUROFIBROMATOSIS OF THE APPENDIX IN VON RECKLINGHAUSEN'S DISEASE

A Report of a Case

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Merck, C. & Kindblom, L.-G. Neurofibromatosis of the appendix in von Recklinghausen's disease. A report of a case. Acta path. microbiol. scand. Sect. A, 83: 623–627, 1975.

A case of neurofibromatosis of the appendix in a patient with von Recklinghausen's disease complicated by acute appendicitis is described. The possible pathogenetic relationship between neurofibromatosis and the leiomyomatous hyperplasia found in this case is discussed.

Key words: Neurofibromatosis; appendix; case report.

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Gastrointestinal manifestations of von Recklinghausen's neurofibromatosis are rare. Almost all published cases report involvement of the small intestine, the stomach or the rectum (*Barber & Hanna* 1959, *Westen et al.* 1963). In only a few cases was the large intestine involved (*Raszkowski & Hufner* 1971). Since patients with gastrointestinal neurofibromas are usually symptom-free it has been suggested that neurofibromas of the gastrointestinal tract are probably more common than is generally believed (*Levy & Khatib* 1960, *Westen et al.* 1963, *Lukash et al.* 1966, *Raszkowski & Hufner* 1971).

The aim of the present report is to describe a case of von Recklinghausen's neurofibromatosis with involvement of the appendix discovered at appendectomy.

CLINICAL REPORT

The patient was a 24-year-old man with no family history of von Recklinghausen's neurofibromatosis. At the age of 16 years an arachnoidal cyst was removed from his left hemisphere. On 4 occasions, plexiform neurofibromas of the eyelid were excised. A single café au lait spot was present on his back. When he presented with abdominal pain of one day duration, he also reported frequent bouts of diarrhea during the previous 3 years. On examination he was slightly febrile. Right-sided abdominal tenderness was elicited and the abdomen was meteoristic. The preoperative diagnosis was acute appendicitis. At exploration, the whole appendix was found to be enlarged and hyperemic and the distal portion gangrenous. The small intestine was dilated and the wall markedly thickened. Appendectomy was performed.

One month postoperatively a radiologic examination of the small bowel revealed a severe narrowing of the distal part of the ileum with a prestenotic dilatation. The radiologic appearance was suggestive of regional enteritis (Crohn's disease). Radiologic examination of the stomach and large bowel produced inconclusive findings and at rectoscopy the mucosa of the rectum appeared normal.



Fig 1 Cross-sections of the appendix showing the thickened mucosa and muscle layers and the prominent nodular structures within the mesenteric adipose tissue.

Fig 2 The mucosa of the appendix with a nodular cobble-stone-like appearance.

PATHOLOGY

Gross Appearance

Macroscopically the appendix was enlarged with fibrin partially covering the serosal surface in which petechial hemorrhages were found and the tip was necrotic. Sectioned transversely the appendix wall showed considerable thickening of the muscle layer (Fig 1) Within the mucosa and submucosa were numerous grey-white, firm, nodular structures which distorted the mucosal surface giving it a cobble-stone-like appearance (Fig 2) Similar nodules were also found in the serosa and the mesentery (Fig. 1)

Histologic Methods

The specimen was fixed in 4 per cent formaldehyde solution, and embedded in paraffin. Five μ thick sections were stained according to the hematoxylin-van Gieson method, with hematoxylin-eosin, and with Masson's trichrome. Alcian blue (Chroma Gesellschaft, Stuttgart Untertürkheim) and toluidine blue (E. Merck, Darmstadt) stainings performed at pH 2.5 and 0.5 and 4 0 and 0.5 respectively were used for the study of heparin sulphate in the mast cells. Luxol fast blue was used to demonstrate the myelin sheaths of the peripheral nerves and Palmgren's silver impregnations method to study the axons. Small pieces of the specimen were embedded in metachrylate and 1–2 μ thick sections were prepared and stained according to the hematoxylin-eosin and hematoxylin-van Gieson method.

Microscopic Appearance

The whole appendix wall and mesenteric adipose tissue was diffusely infiltrated with inflammatory cells, mainly polymorphonuclear leucocytes. This infiltrate was particularly dense in the lamina propria beneath the ulcerated areas.

The nodules seen macroscopically in the mesenteric adipose tissue (Fig 3) in the muscle layer (Fig. 4 5) and in the submucosa and mucosa (Fig 6) were found to be plexiform neurofibromas. The crypts in the mucosa were widely separated by the plexiform neurofibromatous tissue. The neurofibromatous structures consisted of collagen fibers interspersed with small fibroblasts and larger wavy spindle-shaped cells containing elongated central nuclei. The latter cells were shown by Luxol fast blue staining to contain myelin and by Palmgren's silver staining method to possess occasional axons. Numerous mast cells which stained metachromatically with toluidine blue and were positively stained with Alcian blue at pH 0 5 were present within the lesions. Amongst the hyperplastic smooth muscle fibers were several clusters of ganglion cells (Fig. 4 5)

Fig 3 Characteristic plexiform neurofibromatous structures with the external muscle layer of the appendix and in the mesenteric adipose tissue. Masson's trichrome × 25

Fig 4 The external muscle split up by neurofibromatous structures containing clusters of ganglion cells. H & E × 90.

Fig 5 A cluster of ganglion cells within a neurofibromatous structure in the external muscle layer H & E × 160.

Fig 6 Neurofibromatosis of the mucosa tending to form a plexiform pattern. H & E × 160.




4


5


6

DISCUSSION

It is well-known that acute appendicitis can occur secondary to appendix lesions such as mucocele (*Carleton* 1955 *Wesser & Edelman* 1961) adenocarcinoma (*Mauritzen* 1958, *Steinberg & Cohn* 1967) and carcinoid tumor (*Farringer & Tarandis* 1964 *Moertel et al.* 1968) The coexistence of acute appendicitis and peripheral nerve tumors of the appendix has not, as far as we know been reported previously The thickened wall and the severe narrowing of the lumen in the distal part of the ileum in the present case could very well be manifestations of neurofibromatosis. It is probable that the abdominal pain and long standing diarrhea reported by the patient were due to the changes in the small bowel. Gastrointestinal manifestations of neurofibromatosis are rare (*Levy & Khatib* 1960 *Westen et al.* 1963 *Lukash et al.* 1966 *Raszkowski & Hufner* 1971) Neurofibromatosis of the gastrointestinal tract has been known to present itself in the following ways by hemorrhage, perforation of the bowel, intussusception and intestinal obstruction (*Klatsch et al* 1951 *Butler & Hanna* 1959 *Ghrist* 1963 *Staple et al.* 1964 *Donnelly et al.* 1969 *Raszkowski & Hufner* 1971)

Tumors of peripheral nerves, in patients with or without von Recklinghausen s disease have occasionally been reported to contain tissue components other than those normally found in peripheral nerves (*Woodruff et al* 1973) For example, rhabdomyosarcomatous differentiation has been observed in peripheral nerve tumors, these tumors have been referred to as "Triton" tumors (*Masson* 1932) Occasionally other tissue components of mesenchymal origin have also been found in neurofibromatous tumors (*Orlandi* 1895 *Adair et al* 1932, *Stout & Murray* 1942 *Moore* 1942 *Inglis* 1950 *D'Agostino et al.* 1963 *Greene et al* 1974 *Woodruff et al.* 1973) Leiomatous hyperplasia has been observed in arteries in patients with von Recklinghausen s disease and intestinal leiomyomas have also been reported (*Lukash et al* 1966) It has been stated that neurofibromatosis is a hamartomatous disorder in which malformations of tissues other than neuroectodermal tissues may be present (*Willis* 1962) Thus, the marked leiomyomatous hyperplasia seen in the present case could be a feature of such a developmental disorder

The appearance of ganglion cells was a prominent feature in this case of neurofibromatosis of the appendix. Ganglion cells have previously been demonstrated in neurofibromatosis of the large bowel and referred to as ganglioneurofibromatosis (*Donnelly et al.* 1969) As early as 1923 a case of "ganglioneuromatose" of the appendix (*Schult.* 1923) was described which showed a "sehr kräftig entwickelte Muscularis" along with numerous ganglion cells and neurofibromatous structures. We feel that other conceivable explanations of the muscle hyperplasia in the present case could be the stimulation of the muscle either by the ganglion cells or by the physical presence of the longstanding tumor gradually occluding the bowel lumen and thereby invoking muscle hyperplasia.

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EFFECTS OF SUPPLY AND WITHDRAWAL OF FLUORIDE

Experimental Studies on Growing and Adult Rabbits.

8. Phosphatase Activity in Fluorotic Bone

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Rosenquist, J. B. Effects of supply and withdrawal of fluoride. Experimental studies on growing and adult rabbits. 8. Phosphatase activity in fluorotic bone. Acta path. microbiol. scand. Sect. A, 83: 628–632, 1975.

Growing rabbits were given 200 ppm of fluoride in the drinking water during 14 weeks. During this period excessive fluorotic changes developed in the diaphyses of the femur and tibia. The alkaline phosphatase and pyrophosphatase activities increased simultaneously in the fluorotic bone whereas the ratio between the activities remained constant. Acid phosphatase activity also increased. The increase in enzyme activities was regarded as due to the increased bone turnover and not as a primary consequence of fluoride ingestion.

Key words: Fluorosis; acid and alkaline phosphatases; alkaline pyrophosphatase.

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Fluoride has been shown to affect many enzymes *in vitro* (cf. *Venkateswarlu* 1970), i.a. to inhibit the activity of phosphatases (*Freiman et al.* 1962) and pyrophosphatases (cf. *Venkateswarlu* 1970). However, in short term *in vivo* studies on bone, fluoride administration has not been found to depress alkaline phosphatase (*Messer et al.* 1973) or alkaline pyrophosphatase activities (*Sperto et al.* 1969). On the other hand, cattle suffering from chronic skeletal fluorosis was found to have an increased activity of alkaline phosphatases in the fluorotic bone (*Müller & Shupe* 1962).

As skeletal fluorosis is characterized both by excessive formation of irregular bone and increased bone resorption, enzymes participating in bone metabolism may be affected by fluoride and thus contribute to the altered bone morphology. In the present investigation, the activity of some enzymes regarded to be involved in bone metabolism was studied in normal bone and in bone showing morphological features of fluorosis. Bone fluorosis was obtained by a fluoride supplement of 200 ppm to growing rabbits during 12 weeks.

MATERIAL AND METHODS

Fifteen growing rabbits of both sexes, aged 7 weeks at the beginning of the experiment, were fed a commercial diet (Astra-Ewos, Södertälje, Sweden) containing 12 ppm of fluoride. Ten rabbits constituting the experimental group received 200 ppm of fluoride in their drinking water during 14 weeks, while the other 5 rabbits, serving as controls, had distilled water. The drinking water was supplied *ad libitum* and distributed in plastic cups.

After 14 weeks the rabbits were killed by an overdose of sodium pentobarbital. Each tibia and femur was freed from adhering soft tissues. The metaphyses were cut off the bone marrow was removed by a sharp blast of air and the bones were rinsed in saline. Then the bone pieces were frozen in propanol immersed in liquid nitrogen. They were freeze dried in vacuum to constant weight and pulverized at −20 C in a Wiley Laboratory *Mill (Intermediate Model)* provided with 60-mesh sieve and mounted in a freezer.

For morphological documentation, samples from one of the experimental rabbits were dehydrated in absolute alcohol and embedded in methyl methacrylate. Cross sections were sawn from the middiaphyses of femur and tibia and ground down to a final thickness of 80–100 μ. Microradiography was then performed as described earlier (*Rosenquist* 1973 b)

Fluorid Analysis

About 30 mg of bone powder was ashed in platinum crucibles overnight at 700 C. The fluoride determination was done on about 10 mg of ash as previously described in detail (*Rosenquist* 1973 a)

Enzyme and Protein Determination

The bone powder was homogenized for 15 minutes in saline with 0.1 per cent Triton X 100 using a glass homogenizer placed in an icebath. After centrifugation, aliquots of the supernatant were subjected to enzyme and protein determination.

Alkaline phosphatase activity was determined in 0.3 M 2-amino-2-methyl-1-propanol (AMP) buffer at pH 10.8 with di-sodium-alpha naphtyl phosphate as a substrate (*Moss* 1966). The absorption of liberated alpha naphtol was recorded in a spectrophotometer at 335 μ.

Using the same substrate, *acid phosphatase* activity was determined in acetate buffer at pH 5.0. The recording was made in the same way as for alkaline phosphatases.

Alkaline pyrophosphatase activity was determined in 0.2 M Tris buffer at pH 8.5 with sodium pyrophosphate as a substrate. The released phosphate was measured using the method of *Fiske & Subbarow* (1925) as described by *Augustinsson* (1966).

The amount of total *protein* was recorded according to *Lowry et al.* (1951) as described by *Augustinsson* (1966).

Statistics

The significance of differences between the groups was tested by the t-test.

RESULTS

During the observation period two rabbits died of infection, one from each group. The surviving control animals remained healthy. The experimental animals lost appetite, their furs lost lustre and they were increasingly irritated when handled.

Gross Morphological Changes

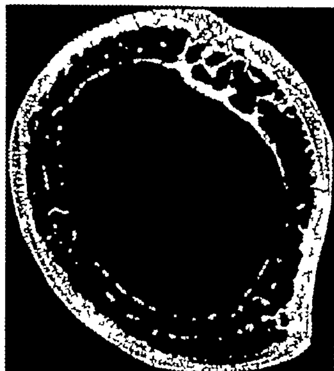
The control bones were smooth and slender in contrast to the experimental bones which were thicker with rough periosteal surfaces.

TABLE 1. *The Fluoride Concentration and the Activity of Alkaline Phosphatase, Acid Phosphatase and Alkaline Pyrophosphatase in Homogenates of Diaphyseal Bone from Femur and Tibia of Normal and Fluorotic Rabbits*

	Fluoride % of ash	Alk. phosph.	Acid phosph.	Alk. pyrophosph.	Alk. pyrophosph. / Alk. phosph.
Femur					
Normal	0.06 ± 0.02	0.620 ± 0.202	0.001 ± 0.003	0.095 ± 0.018	0.119 ± 0.024
Fluorotic	1.42 ± 0.15***	2.763 ± 1.557**	0.015 ± 0.015	0.238 ± 0.077***	0.114 ± 0.091
Tibia					
Normal	0.06 ± 0.01	0.509 ± 0.210	0 ± 0	0.064 ± 0.025	0.153 ± 0.036
Fluorotic	1.43 ± 0.16***	2.945 ± 1.691**	0.034 ± 0.043*	0.277 ± 0.053***	0.113 ± 0.042

* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $0.001 > p$

The enzyme activities are expressed as μmoles liberated alpha naphtol per mg protein per hour (alkaline and acid phosphatases) and μmoles liberated phosphate per mg protein per hour (alkaline pyrophosphatases).





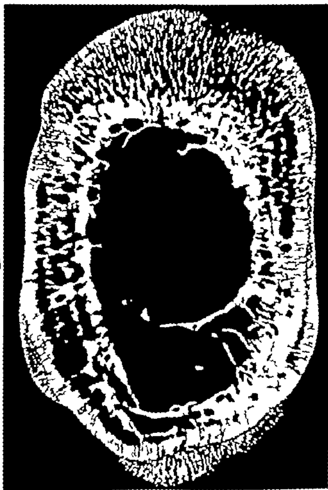


Fig 1 After 14 weeks on a fluoride supplement of 200 ppm, excessive fluorotic changes developed both in femur (left top) and tibia (left bottom) characterized by bone formation and bone resorption. If compared with normal femur (right top) and tibia (right bottom) in the same magnification (× 9.6) it appeared that almost all bone in the fluorotic femur and tibia had been formed during the fluoride regimen.



The tibia was the site of exostosis formation while the femur was more uniformly broadened. A comparison with normal bone revealed that most of the fluorotic bone must have been formed during the observation period (Fig 1)

Fluoride Analysis

The fluoride concentration of the fluorotic bones was more than 23 times higher than that of the controls (Table 1) Within the control and experimental groups, respectively the fluoride concentrations in tibia and femur were similar

Enzyme Analysis

With respect to the activities of the different enzymes there were no differences between the two bones within each group (Table 1) There was a significantly increased activity of all enzymes in the fluorotic bones as compared with the normal bones. The ratio between alkaline pyrophosphatase and alkaline phosphatase was the same in normal and fluorotic bone.

DISCUSSION

Fluoride supplement of 200 ppm in the drinking water caused skeletal fluorosis with profound alterations of the bone morphology characterized by both excessive bone formation and bone resorption. The activities of some enzymes regarded to participate in the bone remodelling processes were significantly increased as compared with normal bone The simultaneously increase both in alkaline phosphatase and alkaline pyrophosphatase activities, the ratio of which was the same in normal and fluorotic bone, suggests that neither enzyme has a specific sensitivity to fluoride. The constant ratio between the activities would rather indicate that they are manifestations of the same enzyme as has been suggested i.a. by *Wöltgens et al.* (1970) in a study of rat molars.

The activity of alkaline phosphatase in calvaria was not found to change during a short term study (one week) of new-born mice given 50 ppm of fluoride in the drinking water (*Messer et al* 1973) This comparatively moderate dose was chosen in order that toxic effects might be escaped. Even doses as large as 800 ppm of fluoride in the food for one week did not change the activity of alkaline pyrophosphatase in femora of new-born chickens (*Spierto et al.* 1969) Thus, the fluoride did not seem to exert any direct effects upon these enzymes activities. Any morphological examinations were not done in the two cited studies.

The findings in the present investigation, *viz.* the unaltered mutual relationship between the two alkaline phosphatases, and the findings by *Spierto et al.* (1969) and *Messer et al* (1973) *viz.* no change in phosphatase activity due to short term ingestion of fluoride, indicate that the increased enzyme should mainly be attributable to the increased bone turnover It is hardly a primary effect of fluoride ingestion.

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CHRONIC RHEUMATIC VALVULAR HEART DISEASE

An Autopsy Study

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Hallgrímsson, J. Chronic rheumatic valvular heart disease. An autopsy study. Acta path. microbiol. scand. Sect. A, 83: 633–638, 1975.

The incidence of chronic rheumatic valvular heart disease in Iceland was investigated via autopsies performed from November 1965 through December 1974. During this period, approximately 12.400 Icelanders died at the age of 16 years and older and 28.8 per cent of these are included in the study. At autopsy, males were found to outnumber females by 2:1. Rheumatic valvular heart disease was found in 38 subjects, i.e. in 1.06 per cent of the cases, the sex distribution being 0.67 per cent males and 1.83 per cent females. As regards the hearts with chronically deformed valves, the deformity was of rheumatic origin only in 20 per cent of the cases; the majority, or 69 per cent, presented calcified aortic stenosis. Hospital records applying to most of the subjects were available and according to these, only 18 per cent disclosed a history of rheumatic fever. The diagnosis of rheumatic valvular disease was first established at autopsy in 71 per cent of the cases. An evaluation of the functional derangements of the diseased valves was not attempted, but there is reason to believe that many of the lesions were too mild to provoke significant symptoms and signs. Previous doubts about the existence of rheumatic heart disease in Iceland have been settled in this study.

Key words: Rheumatic heart disease; autopsy study.

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Two separate studies of the incidence of chronic rheumatic valvular heart disease in Iceland have lead to different conclusions. The first was based on autopsies and indicated that the disease hardly existed (3). The second was a clinical study, partly supported by autopsies, which suggested that the incidence was only a little lower than that in Denmark (9).

In order to settle the controversy hearts derived from a series of autopsies performed in the Department of Pathology at the University of Iceland were studied. All cases of chronic valvular heart disease were recorded and subdivided into three groups according to the origin of the valvular damage, i.e. rheumatic, degenerative and congenital.

This report is concerned with the observed cases of chronic rheumatic heart valves. The two other types of valvular deformities will be a subject of a separate report.

MATERIAL AND METHODS

Late in 1974 the total population in Iceland was approximately 215.000. Slightly more than fifty per cent of the population was concentrated in Reykjavik and in communities close to it. Accordingly

the largest hospitals are located in Reykjavik and they accommodate patients from this area as well as from many other parts of the country where adequate hospital facilities are lacking.

This concentration of medical facilities has made it possible for the Department of Pathology at the University of Iceland to serve as the sole institute for anatomical pathology.

The autopsy cases are received from the hospitals in the Reykjavik area together with those to be subjected to forensic autopsy, the latter coming from all parts of the country.

The period of investigation covered 9 years and 2 months, *viz.* from November of 1965 through December of 1974. During that time about 13,200 Icelanders died; 4453, or 33.7 per cent, were autopsied in the Department of Pathology. This series included newborns, but stillborns were excluded. Since the purpose of the investigation was to establish the incidence of chronic valvular heart disease, persons who died at the age of 16 years and older were selected, *i.e.* a total of about 12,400. The material comprised the hearts from 3578 of these, corresponding to 28.8 per cent of all who were at the same age at the time of death.

The sex ratio of subjects autopsied during the period was 2 males to each female, which means that approximately 40 per cent of the males and 25 per cent of the females in the age groups concerned who died are included in the study. The mortality statistics applying to the country during the same period show that the male/female ratio is 1.2/1.0. There are two main reasons why the sex ratio in autopsy and mortality statistics differ. In the first place, a considerable number of the autopsies were medico-legal autopsies, the majority of which involved males who, as is well-known, are more prone than females to die in accidents, or suddenly and unexpectedly at a relatively young age. Secondly, during the period of investigation there was a decreasing number of autopsies of subjects from old-age homes where the majority of residents usually are females. The value of the study is somewhat depreciated by the sex ratio which is not representative of the population of the country.

The study is based on the author's personal observations, either during the performance of autopsies or more often, after completion of the autopsies when the formalin fixed hearts were inspected after they had been opened by conventional cuts through the valves. The diagnosis was primarily established on the basis of the gross appearance of the heart valves and by palpation by which the pliability was determined.

The following criteria for the selection of rheumatic hearts were set up (4):

Aortic valve. Interadhesion between aortic cusps at two or at each of the three commissures with rigid, fibrotic and sometimes calcified cusps.

Mitral valve. Interadhesion between the leaflets thickened by fibrosis and, occasionally, calcification; thickened, shortened, and sometimes fused chordae.

Tricuspid valve. Same as the mitral valve.

The mitral valve chordae were thickened and shortened in three hearts but interadhesions between the leaflets were insignificant. A microscopic examination revealed thick-walled blood vessels in the leaflets which also were involved with fibrosis and, accordingly, these hearts were considered to be rheumatic.

The morphological criteria were judged to be inadequate for an accurate determination of the types of functional disturbance of the valves, *i.e.* whether there was primarily stenosis, an insufficiency, or combinations of both. Grading of the lesions according to severity was not attempted. Most of the subjects who at autopsy were found to have rheumatic heart valves had been patients in hospitals in the Reykjavik area, either at the time of death or earlier. Hospital records were therefore available, including data on the previous history, physical examination and clinical diagnosis. In four cases subjected to medico-legal autopsy nothing but the police records relating to the death was available.

The number of deaths officially recorded to be due to rheumatic valvular heart disease was obtained from the public health reports in Iceland. The Statistical Bureau of Iceland supplied the mortality statistics.

TABLE 1. *Deformed Heart Valves at Autopsy of 3578 Icelanders Aged 16 Years and Older Classified according to Sex and Type of Lesion*

	Males	Females	Total	% of autopsies	% of deformed valves
Calcific aortic stenosis	76	54	130	3.69	69
Congenital deformity of aortic valve without secondary changes	17	4	21	0.59	11
Rheumatic valvular disease	16	22	38	1.08	20

TABLE 2. *Blood Vessels in Valves of 22 Rheumatic Hearts*

	Number of hearts	Blood vessels	
		Aortic valve	Mitral valve
Isolated aortic valve disease	11	7	2
Isolated mitral valve disease	5	3	5
Combined aortic and mitral valve disease	6	4	5

TABLE 3. *Distribution of Rheumatic Hearts according to Valves Involved and Sex of Patients*

	Males	Females	Total	%
Isolated aortic valve	9	4	13	34.2
Isolated mitral valve	4	8	12	31.6
Combined aortic and mitral valves	3	9	12	31.6
Combined mitral and tricuspid valves		1	1	2.6

RESULTS

A total of 169 hearts with deformed valves were found among the 3578 examined (Table 1). Rheumatic valves were found in 1.08 per cent, all were of the chronic type, and none of these were considered to be in an active phase. The sex distribution of the rheumatic hearts was 16 male hearts versus 22 female. If, however, the unequal representation of the sexes in the material is taken into account, the incidence is 0.67 per cent in the case of males and 1.85 per cent in the case of females.

Microscopic examination of the aortic and mitral valves from 22 of the 38 rheumatic hearts revealed thick-walled blood vessels in 17 (Table 2). Most valves with macroscopic evidence of rheumatic changes contained such vessels, but the latter were also found in the grossly uninvolved valve in several hearts considered to be affected by mono-valvular disease, probably indicating an earlier rheumatic inflammatory change.

The valvular distribution of the rheumatic changes appears from Table 3. Hearts with trivalvular involvement were not observed. In three hearts, valvular lesions other than rheumatic were found. In two females with rheumatic aortic valves calcified mitral annuli were also present. In one male with a rheumatic mitral valve, an interadhesion between two aortic valve cusps was observed the cusps otherwise being thin and soft with no apparent fibrous or calcification of the involved commissure. This aortic valvular deformity was therefore considered to be of congenital origin and of the same type as deformities of the 21 hearts included in Table 1.

Only 7 patients, or 18 per cent, had a past medical history of rheumatic fever. In the case of four other patients, clinical diagnosis of rheumatic valvular heart disease had been established, though a history of rheumatic fever was absent. Thus, the diagnosis had been established before death in the case of 11 out of 38 patients, i.e. in 29 per cent of the cases. As regards the remaining 27 patients, the diagnosis was not recorded at the time of hospitalization in 21 cases and thus, it may

TABLE 4. *The Presence and Absence of Heart Murmurs in 21 Patients with Undiagnosed Rheumatic Valvular Heart Disease Classified according to Valves Involved*

	Murmur	No murmur
Isolated aortic valve	4	4
Isolated mitral valve	3	4
Combined aortic and mitral valves	4	2

TABLE 5. *Approximate Mean Age of Patients With Rheumatic Hearts at Death Classified according to Valves Involved and Sex of Patients*

	Males	Females	Both sexes
Isolated aortic valve	65	72	67
Isolated mitral valve	72	69	70
Combined aortic and mitral valves	58	70	67
All rheumatic hearts	65	70	68

not have been established. In more than half of these however physical examination had revealed a heart murmur (Table 4)

Fig 1 shows graphically the 32 rheumatic hearts found at autopsy and the 33 rheumatic hearts recorded in the mortality statistics for the years 1966–1973 The difference in the individual years is considerable.

The mean age of patients at the time of death appears in Table 5 The youngest male was 38 years old and the oldest 87 whereas the youngest female was 53 and the oldest 91 years old.

Among the 38 cases, 8, or 21 per cent, were subjected to medicolegal autopsy because of sudden and unexpected death or death in an accident. In three of these, falls were responsible for head injuries which lead to death and the other 5 had died suddenly at home.

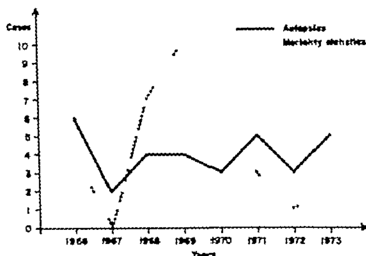
DISCUSSION

It can hardly be doubted that the improved standards of living combined with the preventive and curative measures introduced in the last decades have reduced the incidence of rheumatic heart disease in many countries (7 12) At the same time, health screening and more refined diagnostic methods have brought many cases into light which hitherto would not have been noticed or diagnosed. The present morphological study reveals clearly that clinical methods still are inadequate for the detection of many such cases.

Morphologically a chronically deformed rheumatic mitral valve is generally easy to recognize, but a deformed aortic valve is much more difficult to evaluate, as pathological changes other than rheumatic changes are common findings in these cases. In countries where standards of living and health care are high, most aortic valve deformities are probably of degenerative nature, either secondary to a malfunctioning, congenitally deformed valve or due to excessive wear and tear of an otherwise normally formed tricuspid aortic valve. The study presented here is in support of this theory Anyhow it must be realized that a deformed rheumatic valve secondarily may become involved in a degenerative process, much in the same way as the congenitally deformed valve. Therefore there are instances in which it may be impossible to distinguish rheumatic changes from other pathological changes.

This study confirms the clinical observation that rheumatic heart disease exists in Iceland (9) The conclusion drawn in an earlier report according to which the disease probably did not occur in this country must be disregarded (3) However the lesion must be considered to be rare as compared with the frequency in other countries. In the years 1941–1955 the incidence observed at autopsies performed in Bergen was 4 01 per cent, and in the years 1954–1955 it was 5.94 per cent in Oslo, all age groups except stillborns being included (11) Calculations on the same basis point to an incidence of 0.84 per cent in Iceland. There is a difference of 20 years between the Norwegian and the Icelandic studies and, accordingly the figures could be misleading as the incidence in Norway may have declined since then, although any decrease during the actual period of study could not be noted (11) In the years 1959–1960, the incidence of rheumatic heart disease ob-

Fig. 1. A comparison of the number of cases of rheumatic valvular heart disease found at autopsy and registered in the mortality statistics for the years 1966–1973



served at autopsies in Israel on subjects aged 30 years or more was 6.3 per cent and, during the years 1952–1958, it was 3.5 per cent calculations including all age groups from 6 months and older (10).

The incidence of rheumatic heart disease was found to be higher among females than among males and, if adjusted to the sex ratio in the series the disease seems to be 3 times as frequent in females as in males in Iceland. The frequency was also found to be higher among females in Israel and Norway (10, 11).

The distribution of the rheumatic changes over the isolated aortic valve, the isolated mitral valve, and the combined aortic and mitral valves was found to be almost uniform. If considered in the light of the uneven sex ratio in the series it might be postulated that the distribution of isolated aortic valve lesions is even among the sexes in Iceland, whereas isolated mitral and combined aortic and mitral valve lesions are up to four times as common in females as in males. However such small numbers of cases do not warrant a statistical analysis in this respect.

An isolated aortic valve stenosis is by some investigators considered to be either of senile nature or of congenital origin (6). Others, who accept a rheumatic origin of an isolated aortic valve stenosis, have found the incidence to be lower than that of isolated mitral valve or combined valvular disease. Thus in a series of 4800 autopsies performed in Boston Cook and White found that combined aortic and mitral valve disease was the most common lesion (46 per cent), followed by isolated mitral disease (27 per cent), trivalvular disease (16 per cent) and isolated aortic valve disease which was present in only 5 per cent of the rheumatic cases. In Malmö, Hall found at autopsy isolated mitral valve disease in 57 per cent of the cases, a combined aortic and mitral valve disease in 24 per cent, and an isolated aortic valve disease in 17 per cent of rheumatic hearts (5). An explanation of the difference in valve distribution observed in these three studies in Iceland, the United States, and Sweden, respectively, may be that the morphological criteria differed, but other factors such as an inherent variation in susceptibility to the rheumatic inflammatory process may also play a part.

The diagnosis of rheumatic valvular disease was first established at autopsy in 71 per cent of the Icelandic cases, a percentage which is considerably higher than that found elsewhere (1, 11).

Several explanations are available why this large number of cases remained clinically undetected. In the first place, the fact that a previous history of rheumatic fever frequently was absent could have lead to a misinterpretation of heart murmur. A negative history

of rheumatic fever is said to be more common in the older age groups. The mean age at death of these Icelanders was 68 years, an age which is much higher than that observed in Norway where it was 52–53 years (11) Secondly a heart murmur was not heard in several of these patients (Table 4) Silent rheumatic valvular heart disease is well known though it is considered to be a rare occurrence (8) Thirdly four of the subjects had no hospital records. Fourthly in many of the cases the pathological changes were not of a degree sufficient to cause severe valvular deformities. Therefore the functional derangements of these valves may not have been sufficiently pronounced to be detected at routine physical examination.

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REGIONAL DISTRIBUTION OF DMBA INDUCED MAMMARY TUMOURS IN THE RAT

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Torgersen, O Regional distribution of DMBA-induced mammary tumours in the rat. Acta path. microbiol. scand. Sect. A, 83 639–644 1975

Since human mammary cancers have a preference for certain regions of the breast, registration of the topography of DMBA-induced mammary tumours in rats was performed. This revealed that most of the tumours occurred in the upper or thoracic, mammary glands. While the number of tumours in the two abdominal mammary regions was found to be rather constant, the incidence in the two thoracic regions had a tendency to mutual variations. A regional registration should be made in all experiments dealing with induced mammary tumours.

Key words Mammary tumours DMBA-induced regional distribution.

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In human mammary cancer there is a peculiar predilection for the upper lateral quadrant where 30 to 48 per cent of the tumours are seen to occur On the other hand, the lower medial quadrant seems to be relatively spared since only 4.5 to 6 per cent of the cancers are found in this area (3, 5, 7 8, 11 16) A second, possibly pre dilected, site is the central region where some authors have recorded a rather high tumour incidence (11) The careful record by *Haagensen* (7) is seen in Fig 1

Although the mass of mammary tissue contained within the upper lateral breast quadrant may be somewhat larger than that at other sites, this is hardly sufficient to explain such findings. The small and multiple breasts of most laboratory animals do not lend themselves to direct experimental investigations of this problem. On the other hand, since the thoracic and abdominal mammary glands seem to differ in multimammary animals, e.g with regard to blood and lymph vessels (4 17) it was decided to see whether such dif ferences might influence the local incidence of mammary tumours induced by i.v administration of a carcinogen.

4

MATERIALS AND METHODS

Animals. Female *Sprague-Dawley* rats, aged 40 days, were obtained from *Møllegaard Hansens Avlslaboratorier* Ejby Denmark. The animals were kept in clean, wire-bottomed cages, 5 rats in each. They received a well-balanced diet (Food pellets no 153, *Møllecentralen 1/S* Oslo) and water *ad libitum*

Carcinogen At day 55 30 animals were injected intravenously with DMBA (9 10 dimethyl-benzanthracene, *Sigma Chemical Comp Inc*) This was dissolved in soya oil to a concentration of 30 mg/ml by way of ultrasound and incubation over night at 37 C. The concentration of DMBA was adjusted by the addition of 10 per cent Intralipid (*Nyco Laboratories*, Oslo) and repeated use of ultrasound, or violent shaking, to obtain a com-

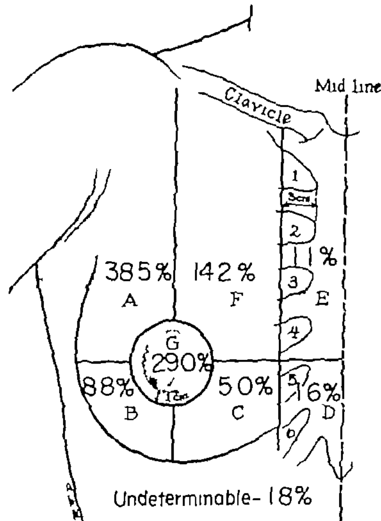


Fig 1 Distribution of cancer in the female breast. According to *Haagensen* (7) Printed with the permission of the author and W B. Saunders Co.

plete emulsion containing 5 mg of DMBA per ml. Thus, intravenous injection of 0.8 ml of this emulsion resulted in an administration of 4 mg DMBA pro animal

Mammary regions. In rats, the thoracic and the abdominal (or abdominal-inguinal) groups of mammary glands can easily be distinguished as four regions, each containing three visible mammary glands (*Fig.* 2)

Tumour registration. Tumours developing in the different regions were recorded once a week during the first twelve weeks, thereafter once a month. The animals were allowed to live until death was imminent, but systematic registration of tumour incidence was terminated after 10 months.

Statistical calculation. Since most of the mammary tumours developed before the 6th month, this date was chosen for statistical evaluation. At this time, all experimental animals were still alive (Fig 4) An approximate method described by *Quenouille* (12) was used. The method is a modification of the *Student's t-test* for the difference of two means. In addition, *Wilcoxon's* signed-rank test for paired observations was used.

Histology Tumour specimens were fixed in 10 per cent formalin and the slides were stained with haematoxylin-eosin.

RESULTS

Six months after I.V. administration of DMBA to 30 rats, a total of 169 mammary tumours were recorded. Among these 112 occurred in the two thoracic mammary regions, whereas only 57 tumours developed in the two corresponding abdominal regions

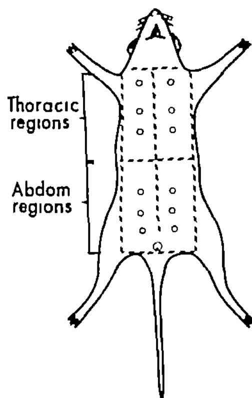


Fig. 2 Mammary regions adopted in the present study

behaved similarly and metastases was not produced by any of these.

DISCUSSION

The use of DMBA for the early production of mammary tumours was introduced by *Huggins et al* in 1961 (6) Since then, the method has been adopted by many writers, but the possibility of a regional difference in tumour incidence has apparently not been mentioned earlier Accordingly some previous experiments may have to be re-evaluated on this basis. In earlier experiments, the three left-sided abdominal mammary glands in several groups of rats were irradiated prior to DMBA-administration (15) At that time we were not aware of the "spontaneous" local variation in DMBA-induced mammary tumours. Since nothing but the three contra lateral abdominal mammary glands were used as controls, however the results may still be valid.

Any clear-cut explanation of the "thoracic factor" cannot be offered at present. In mam

(Fig. 3) This difference is significant ($p < 0.001$ by the *Student's* t test and $p = 0.01$ by *Wilcoxon's* test, both two-tailed tests) The tumour incidence was slightly higher in the right thoracic region than in the left region (60 versus 52 tumours) whereas the number of tumours was almost identical in the two abdominal mammary regions (28 versus 29) It applies to the thoracic as well as the abdominal region, that the incidence of tumours was highest between the 6th week and the 6th month (Fig. 4) Fig 5 shows how the tumours are distributed in the different animals, and Fig. 6 shows the cumulated tumour curves. Both Fig. 5 and Fig 6 show a clear preponderance of tumours in the thoracic regions.

Macroscopical examination of the tumours revealed histological findings similar to those described elsewhere (1 2, 10, 13) In spite of the varied histological pattern, all tumours

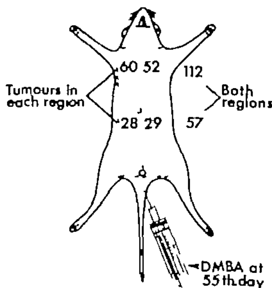


Fig 3 The topographical distribution of mammary tumours 6 months after DMBA.

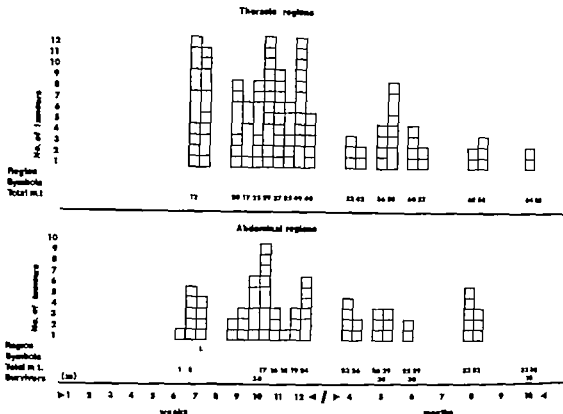


Fig. 4 The incidence of mammary tumours following i.v. DMBA injection. Each square represents one tumour per animal, the rectangular areas signifying the presence of two or more tumours in the same animal.

mals with more than two mammary glands, the glandular mass in the subcutaneous tissue is diffusely spread over the fascia of the pectoral muscles (9). This explains the common finding that DMBA-induced mammary tumours most often appear outside the visible, periareolar area seen with the naked eye. One possibility is that the thoracic mammary glands in rats, developing from the proximal parts of the two milk lists, may have a higher number of glandular ramifications than the glands in the abdominal regions. Although such findings have not been reported in the available literature, this "endogenous" explanation cannot be wholly disregarded at present.

Another possibility is that the increased susceptibility of the thoracic glands to DMBA induced tumours may be of exogenous origin. In multimammary animals, the lymph of the thoracic mammary glands mainly drains to the axillary lymph nodes, and possibly to other thoracic lymph nodes, while the lymph from the abdominal glands mainly passes to the inguinal nodes. Since some of our animals suffered from sneezing and other signs of upper respiratory infection, we have speculated whether some infectious agent (e.g., a virus) might have reached the thoracic mammary glands by retrograde lymphatic extension (from the lower cervical or possibly inner thoracic lymph nodes) thereby predisposing these groups of mammary glands to the action of DMBA. If a similar agent were present in certain human axillary lymph nodes, e.g. following a particular local infection an infective agent might be assumed to have spread to the nearest,

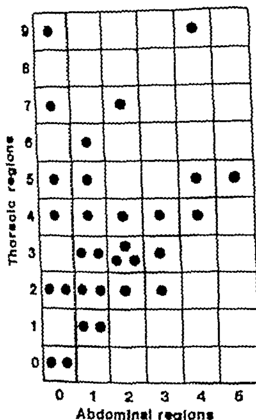


Fig. 5 The distribution over thoracic and abdominal areas of DMBA-induced mammary tumours.

or upper lateral, mammary segment, thereby localizing the action of a circulating carcinogen. Similarly if the human mammary tumour is located in the central region, a slight infection of the nipple, caused by a particular agent, might have exerted a similar effect.

Since the aetiolgy of mammary cancer is largely unknown, this hypothesis may merit further consideration. Further work along this line is in progress.

The author is much indebted to senior lecturer *Rolf Bjerkes* for all work connected with the statistical calculations. Mrs. *Jorunn Sender* kindly prepared the DMBA-solutions used. Thanks are likewise due to Mr. *Th. Lovstad* for technical help with the experiments and care of the animals. The work was supported by the *Norwegian Cancer Society (Landsforeningen mot Kreft* Oslo.)

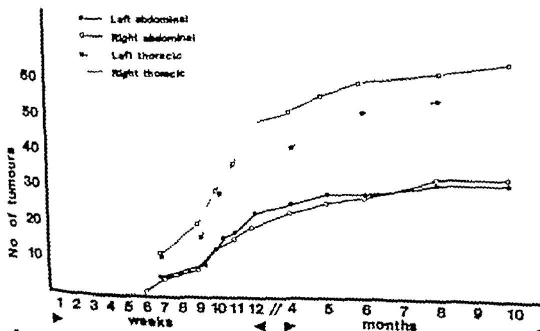


Fig. 6 Curves showing the cumulated number of tumours in the various regions after DMBA.

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MALIGNANT HYPERTHERMIA IN A FAMILY

The Neurophysiological and Light Microscopical Study of Muscle Biopsies of Healthy Members

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Reske-Nielsen, E., Haase, J. & Kelstrup, J. Malignant hyperthermia in a family. The neurophysiological and light microscopical study of muscle biopsies of healthy members. Acta path. microbiol. scand. Sect. A, 83: 645–650, 1975.

12 healthy persons—close relatives to two children who died of malignant hyperthermia—volunteered for biopsies of striated muscle and skin, electromyography and electroencephalography. The investigations of muscle biopsies comprised material for enzyme histochemistry and ordinary light microscopy including visualisation of the intramuscular nerves. Out of 12 clinically healthy persons 9 revealed abnormalities of the muscle fibres, 11 showed degenerative and regenerative alterations in the intramuscular nerves, in EMG 7 turned out to produce slight neuropathy and 7 displayed abnormal EEG tracings. These findings support the idea that the etiological key (or keys) in the peculiar pathophysiological entity of malignant hyperthermia may even be found outside the striated muscle cell.

Key words: Hyperthermia, malignant, familial; muscle biopsy; neurophysiology; light microscopy.

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Clinical and serological studies in a Danish family consisting of 147 members—in which two died of malignant hyperthermia—have been reported earlier (Fig. 1) (6).

This study led us to conclude that there was no obvious pattern of inheritance of the genetic material which leads to malignant hyperthermia during anaesthesia—and that estimation of creatine phosphokinase in serum is but poorly correlated to the probability of developing a symptomatic clinical event as a consequence of an anaesthesiological challenge.

It seemed of major importance to us to try to survey the histological architecture of striated muscle—including the intramuscular nerves—in *healthy* close relatives to patients, who had displayed malignant hyperthermia. The purpose of this paper is to present the results of light microscopic investigations in biopsy material obtained from volunteering members of the family—together with the results of electromyography and electroencephalography in the same persons.

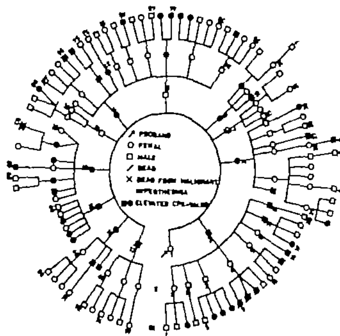


Fig 1 Pedigree of a family consisting of 147 persons in four generations, of whom 12 healthy persons had muscle biopsy performed.

MATERIAL AND METHODS

12 healthy relatives from the family—2 males and 10 females aged 22 to 43 years—volunteered for biopsies of striated muscle and skin, electromyography and electroencephalography (Table 1) These 12 persons were distributed in three family groups consisting of one four and seven persons (Fig. 1 Table 1) The investigations in the muscle biopsies comprised material for enzyme histochemistry electron microscopy ordinary light microscopy—including visualization of the intramuscular nerves. The specimens were taken in this order

The material for enzyme histochemistry (myofibrillary ATP-ase (7) DPNH reductase (2) and phosphorylase (1)) was placed immediately in isopentane permanently cooled by solid carbon dioxide. It was then cut in a cryostat t minus 20 C into 16 µ thick sections

The material for electron microscopy was prepared according to an earlier reported technique (10) Our results are discussed in following paper (11)

The material for the visualization of the intramuscular nerves was prepared ad modum *Koelle* and ad modum *Gelrs* (9)

Specimens of skin were fixed in Bouin fluid and hereafter treated conventionally

The neurophysiological investigations were carried out in the Laboratory of Clinical Neurophysiology Odense University Hospital and included electromyography (EMG) in the deltoid and the abductor digiti V muscles with estimations of the distal motor latency to the hand muscle and motor conduction velocity in the ulnar nerve in the dominant arm—together with an electroencephalographic examination (EEG) which included hyperventilation and sleep tracings.

RESULTS

A. *Light Microscopy*

Light microscopy revealed few but distinctly pathological alterations in the ordinary prepared biopsy material (Table 1) Specimens from 9 out of 12 persons were abnormal. The findings included variations in fibre diameter an increased number of nuclei—lying peripherally or in rows centrally placed in muscle fibres, and scattered hyaline and atrophic fibres. Basophilic or necrotic segments were not seen. One specimen contained small perivascular infiltrations of lymphocytes (Fig 2, 1_{ABCD}) The specimens were compared with a normal biopsy material (9)

TABLE 1 *Light Microscopy of Muscle, Electroencephalography (EEG) and Electromyography (EMG) in 12 Healthy Close Relatives of Two Children (III 56 and III 63) Who Died of Malignant Hyperthermia*

Code	Sex	Age	EEG	EMG	Ordinary muscle biopsy: Hyaline fibres	Ordinary muscle biopsy: Atrophic fibres	Subneural apparatuses: Degeneration	Subneural apparatuses: Increased number	Beaded axons	Intramuscular nerves: Coarse thickenings	Intramuscular nerves: Abnormal end plates	Sprouting
II 16	♀	58	+	+	+	+	+	++	+	+	++	+++
II 27	♀	43	0	0	+	0	+	+	+	+	+	+++
II 28	♂	41	0	0	+	+	+	+	+	+	+	+++
II 29	♀	39	0	0	0	0	+	0	0	+	+	+++
II 30	♀	35	+	0	0	0	+	⊥	⊤	–	–	⊤
II 33	♀	38	+	+	0	0	+	0	+	+	+	+++
II 34	♀	38	+	+	+	0	+	+	+	+	+	+++
II 35	♀	32	+	0	+	0	0	+	+	+	+	++
II 36	♀	30	+	+	0	+	+	0	+	+	+	+++
II 37	♀	28	+	+	+	+	+	+	+	+	+	+++
II 38	♂	26	0	+	+	+	+	0	+	+	+	+++
II 39	♀	22	0	+	+	0	+	0	+	+	+	+++

0: normal findings, +: abnormal findings, ⊤: failed.

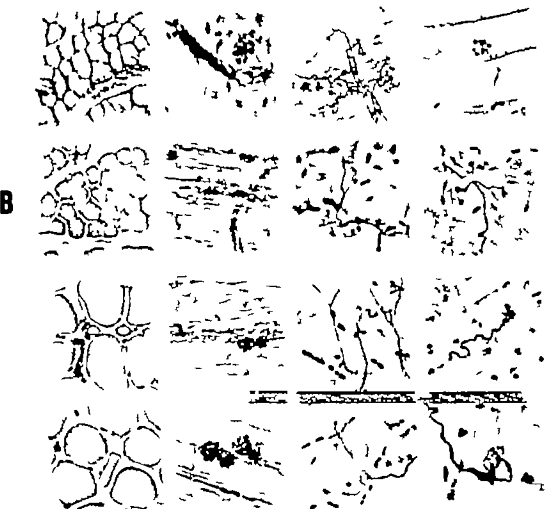


Fig. 2 The collage shows all the light microscopical findings, compared with normal striated muscle. × 125 to 500.

The subneural apparatuses (Table 1) were present in an increased number and few were degenerative with shrunken or poorly defined appearance and abnormally dark staining (Fig. 2, 2ABCD)

The nerve bundles and subterminal nerve fibres (Table 1) displayed irregular thickening and coarse beading in vital methylene blue staining. The motor end plates were either normal in appearance or showed a decreased or increased number of terminal expansions—reflecting the shape of the subneural apparatuses. These expansions could be of varying size being small and dark or swollen. In a few places a motor end plate had normal terminals and a single, dark expansion like a growth cone (Fig. 2, 4ABCD)

Reinnervation was a dominating feature with an abnormal number of double motor end plates—one of which was often very small—collateral ramifications along a subterminal nerve fibre, and tufts of sprouts starting from one point of an axon. Such changes were especially concentrated close to the innervation band (Fig. 2, 3ABCD). Investigations in *enzyme histochemistry* showed no changes with the employed technique.

The skin sections—including the sweat glands—were normal.

B. *Electrophysiological Investigations*

In five persons the *EMG* was normal (Table 1). Six cases displayed a low degree loss of motor units and denervation potentials, and in one case the motor conduction velocity in the ulnar nerve was slightly below the normal values found in our laboratory.

As mentioned above the twelve persons examined belonged to three family groups—consisting of one, four and seven persons.

The one person had a slightly abnormal EEG (Table 1) with short series of 4–7 cps activity in the mid- and pretemporal region accentuated during hyperventilation. In the group of four persons three normal EEG-tracings were found while the fourth displayed sharp waves in the mid- and pretemporal region not accentuated during hyperventilation. In the last group of seven persons two had normal EEG-tracings. Four tracings were slightly abnormal with 4–7 cps activity in the temporal regions with distinct accentuation during hyperventilation. The same tracings were seen in the last member of this group—except that they were more pronounced and showed a tendency for generalization.

Conclusion

Out of 12 clinically healthy persons 9 revealed abnormalities of the muscle fibres, 11 showed degenerative and regenerative alterations in the intramuscular nerves, in EMG 7 turned out to produce slight neuropathy and 7 displayed abnormal EEG-tracings.

DISCUSSION

The enzyme histochemistry of the striated muscle was normal and this is in agreement with *Schiller's findings (12)—the same* technique being employed here. Schiller however found a decreased myofibrillar ATP-ase content in the fibre population when the ATP-ase reaction is changed after passing through Ca-EDTA at a lower pH (4.3–4.6). This investigation has not been performed by us due to an accidental failure of freezing of material stored for this purpose.

The light microscopical examination displayed the same minimal myopathic changes as earlier described (5, 8).

The intramuscular nerves revealed distinctly degenerative and regenerative changes. Similar abnormalities are described in another Danish family with malignant hyperthermia (3). *Harriman* (4) interpreted the collateral ramifications in the motor end plate area as consequences of the myopathy. However, in a later publication he pointed out that the alterations might indicate an associated limited *neural* abnormality (5). This observation is in accordance with the slight neurophysiological alterations in our family. At the same time these findings support the idea that the etiological key (or keys) in the

peculiar pathophysiological entity of malignant hyperthermia may even be found outside the striated muscle cell (11)

The neurophysiological investigations were performed and evaluated by dr *Erik Sindrup* Laboratory of Clinical Neurophysiology Odense University Hospital.

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MALIGNANT HYPERTHERMIA IN A FAMILY

The Ultrastructure of Muscle Biopsies of Healthy Members

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Reske-Nielsen, E., Haase, J. & Kelstrup, J. Malignant hyperthermia in a family. The ultrastructure of muscle biopsies of healthy members. Acta path. microbiol. scand. Sect. A, 83: 651–660, 1975.

Twelve healthy relatives of two children who died of malignant hyperthermia—volunteered to give biopsies of striated muscle. Electron microscopy of their muscles revealed nonspecific myofibrillary degeneration and profound alterations of parts of muscle fibres. Furthermore, constructional faults were found in the architecture of the muscle. Previous investigations correlated with these abnormalities support the assumption, that inborn errors of metabolism may involve membranes of other tissues as well as striated muscle.

Key words: Hyperthermia, malignant, familial; muscle biopsy; ultrastructure.

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The light microscopical investigations were reported (15) in 12 healthy persons from a Danish family of 147 members. Two children of this family died of malignant hyperthermia and clinical and serological studies were published (8, 9) previously. This article reports the electron microscopical findings in the same 12 healthy members who were closely related to the children in whom anaesthesia caused malignant hyperthermia.

MATERIAL AND METHODS

The material is comprised of biopsies from the long palmar muscle of the non-dominant arm in 12 healthy and volunteers whose relation to the deceased children can be seen in table 1 and the pedigree (see the immediately preceding paper). The specimens were fixed in glutaraldehyde and osmium tetraoxide and dehydrated and embedded in Vestopal W. Six grids from each of five blocks of each specimen were examined. The grids were stained with uranyl magnesium acetate and lead citrate and examined in a ZEISS EM 9. Further details of the method were published elsewhere (11, 13). Our findings are compared with the ultrastructure of muscle biopsies from 11 healthy young people (13).

RESULTS

Nearly every grid displayed pathological changes, which were focal and identical from grid to grid.

1. *Myofibrillary Degeneration*

A general feature is reduction in diameter in very varying degree of the muscle fibres

TABLE 1 *Ultrastructure of Muscle and the Osmotic Resistance of the Erythrocytes Digestive-Sc... tives of Two Children (III 56 and II...*

[illegible]	Sex	Age	Osmot. resist. of erythrocytes	S-ATPase	CPK	Degenerative alterations: Myofibrillary disorganization	Myofibrillary degeneration	Osmiophilic bodies	Lysosomes	Myelin bodies	Inclus...
16	♀	38	0	0.26	2.3	+	+	+	+	0	0
27	♀	43	+	0.39	5.6	+	+	+	0	+	0
28	♂	41	+	0.30	15.6	+	+	+	+	+	+
29	♀	39	+	0.29	3.0	+	+	+	+	+	0
30	♀	35	+	0.09	3.5	+	+	0	+	+	[illegible]
33	♀	38	0	0.28	4 1	0	+	+	+	0	[illegible]
34	♀	38	0	0.44	6.0	0	+	+	0	0	0
35	♀	32	+	0.66	5.9	0	+	+	0	0	0
46	♀	30	0	0.62	4.0	+	+	+	+	0	[illegible]
47	♀	28	+	0.73	5.8	+	+	0	0	0	+
48	♂	26	+	0.64	10.0	+	+	+	0	0	0
49	♀	22	+	0.56	4.0	0	+	+	0	0	[illegible]

Osmotic resistance 0: normal + decreased. S-ATPase normal value = 0.51–0.57 CPK normal value = ♂ < 8.2 IU/l, ♀ < 4 4 IU/l. EM 0 normal, + abnormal.

and the myofibrils. Parts of the muscle fibres present displaced A and I-bands and spiky undulating Z-lines (Table 1 Fig. 1) Some muscle fibres are atrophic with thin myofibrils, distorted sarcomeres, and regions of myofibrillary degeneration such regions sometimes merge into areas of total destruction of the muscle (Fig 2)

At higher magnification myofibrillary degeneration is apparent there is loss of filaments of the A and I bands and wide separation of myofibrils. Mitochondria, glycogen and other electron dense granules occur in place of the myofibrils. Myelin bodies, osmophilic bodies and lysosomes lie between the myofibrils and close to the nuclei. In two patients a few tubular aggregates were seen (Fig. 3) The plasma membrane was often folded in places.

2. *Other Structural Abnormalities*

In eight persons profound alterations of whole muscle fibres were observed. Bands of probably A-band and Z material alternated with ribbons of abnormal I-fibrils containing Z-lines (Fig 4) Vacuolated, swollen mitochondria, distended sarcotubular systems and electron dense granules were seen between the widely spaced fibrils. The M-line—and especially the Z-lines—were in places reduplicated and were seen in several contiguous sarcomeres. Both M and Z-lines were sometimes missing Fork-shaped sarcomeres were seen in four persons. The organelles between the abnormal fibrils were as a rule normal. The T tubules were hypertrophic in one person (Table 1)

All the electron microscopical findings are grouped together in Fig 5*

* Identical electron microscopical alterations were found in a muscle biopsy from a six year old boy in another family The biopsy was performed because his little sister had previously died of malignant hyperthermia.

Adenosine Triphosphatase (S-ATPase) and Creatine Phosphokinase (CPK) in 12 Healthy Close Rela-
Who Died of Malignant Hyperthermia

Anomaly of sarcomeres									
A-band		M-line		I-band		Z-line			
Increased length	Missing	Double	Missing	Increased length	Missing	Double	Missing	Extra sarcomeres	Extreme abnormalities
+	0	+	0	+	0	+ +	0	+	0
+	0	+	0	+	0	+ + +	0	+	+
+	0	+	0	+	0	0	0	0	+
+	+	+	0	+	0	+ +	0	+	+
+	0	+	0	+	0	+ +	+	0	+
+	0	+	+	+	0	+ +	0	0	0
+	0	+	0	+	+	+ +	0	0	+
+	+	+	0	+	0	+ +	0	0	+
+	0	+	0	+	0	+ +	0	+	+
+	0	0	0	+	+	0	0	0	+
+	0	+	0	+	0	+ + +	0	0	0
[illegible]	0	0	0	0	0	0	0	0	0

Some A-bands = half normal length.

DISCUSSION

In previous papers (8, 9 15) we reported clinical, serological and light microscopical investigations in healthy relatives to two patients, who died of malignant hyperthermia induced by anaesthesia.

In the present study of the same 12 healthy relatives all the biopsies displayed nonspecific degenerative alterations of the myofibrils (Table 1) Degenerative processes in healthy relatives and in patients with malignant hyperthermia have been found by other authors (1 2, 6, 16) who also regarded them as nonspecific changes. The tubular aggregates are nonspecific alterations which may be seen in healthy young people and in diabetics (12, 13)

In 11 cases other structural abnormalities were seen. The essential alterations of the sarcomeres being the occurrence of double Z- and M lines, which cause secondary increased length of the A and I-bands. The double Z lines were mentioned in a previous paper by one of us concerning malignant hyperthermia (14) Double Z-lines occur seldom but were described in progressive muscular dystrophy (10) and during regeneration (4) Comparing the illustrations by these authors with ours it is evident that the morphology is different. In biopsies from healthy persons in this family with malignant hyperthermia the double Z-lines lie in series and each of them is identical with normal Z-lines, but in muscular dystrophy W G.P Mair describes double Z-lines which are thickened and distorted and not always in series. Double M lines, which lie in series and have the same morphology as in healthy young people (13) to the best of our knowledge have not been described previously Z- and M lines were lacking in one case each. In four cases fork-shaped sarcomeres were seen. All these structural abnormalities are regarded by us *as errors in the architectural design of striated muscle* (Fig 6) We have found these structural abnormalities of the sarcomeres in three families with malignant hyperthermia in respectively one (see note p 652) four (14)



Fig 1 Three fibres are present One reveals streaming of Z-line, running to A-band. One displays mitochondria accumulations. One is normal. Z Z-line; SZ Streaming Z-line N Nucleus A A-band I I-band Mi Mitochondria. × 6000

and 12 persons (15) Similar ultrastructures of the striated muscle in healthy persons seem not to have been described previously We do not accept that electron microscopy of muscle from a clinical point of view can be used to select persons with a high risk of developing malignant hyperthermia.

Conclusion

A study of the literature and our investigation of families with malignant hyperthermia revealed abnormalities of the muscle fibres and the intramuscular nerves at light microscopy (3 15) Furthermore we found decreased osmotic resistance of the red blood cells and decreased digoxine-sensitive adenosine triphosphatase in the membrane of the erythrocytes (5 7) All these alterations reflect together with the abnormal serum creatine phosphokinase an abnormality of the cell membrane. Our series of papers have in our opinion supported the assumption that one or more inborn errors of metabolism might involve membranes in other tissues in addition

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Fig 2 Parts of two fibres are present The left one is normal. The right one shows areas (arrows) with totally destroyed fibrils. In the middle of the illustration the myofibrils are small with spiky and streaming Z-lines. The field marked with → has partly vanished myofibrils continuing in a granular electron dense material. MF Myofibrils Z: Z-line MB Myelin body SZ Streaming Z-line. × 6000

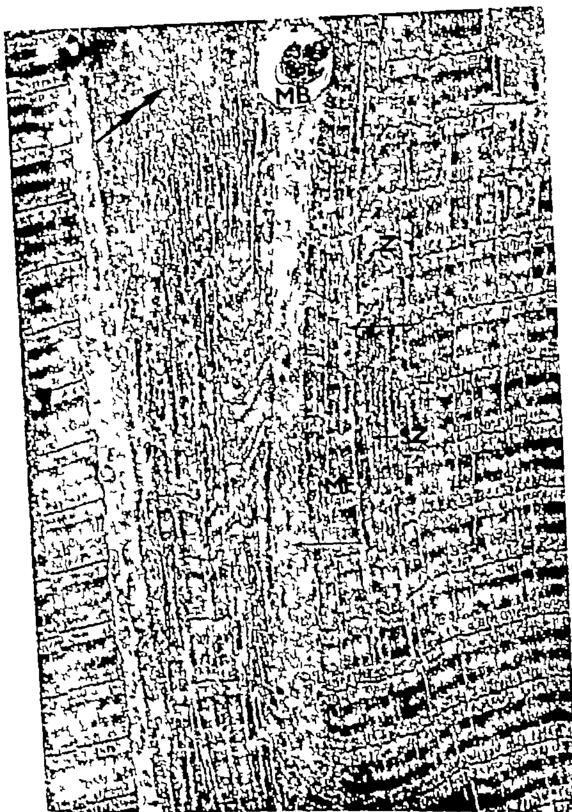

MB
Z
Z
M



Fig. 3 Cross and longitudinal sectioned tubular aggregates. Note the lysosomes. TU Tubules L. Lysosomes MF Myofibril BM Basement membrane. × 27000



Fig. 4 Grid window with total disarrangement of muscle fibres. Bands of probably A-band and Z-lines (arrow) are shifting with bands of abnormal I-band with Z-lines (arrow) AZ: A-bands and Z-lines; IZ: I-bands with Z-lines × circa 2000 Identical iterations named "contraction clump" are described by M. J Cullen and J J Fulthorpe in Duchenne Muscular Dystrophy J neurol Sciences 24 179 200, 1975

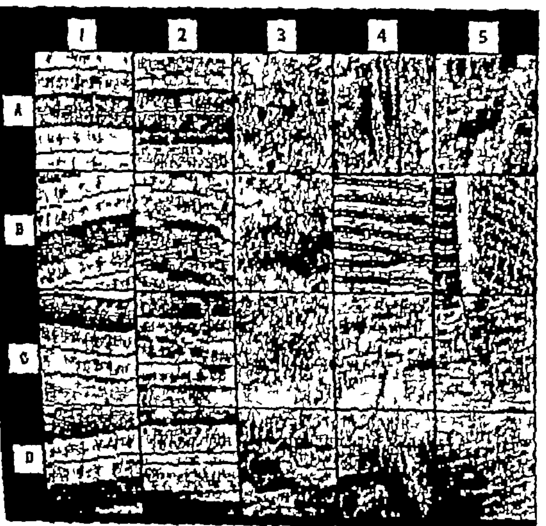


Fig. 5 The collage shows all the electron microscopical findings, compared with a normal striated muscle. The magnifications (× 2000 to 27000) are varying because of different subjects and technical reasons.

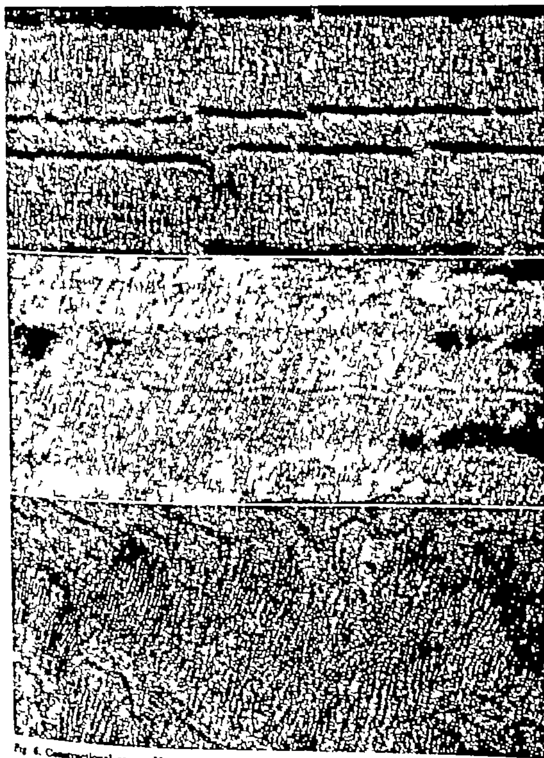


Fig. 6. Constructional errors. Upper: double Z-lines. Middle: double M-lines. Lower: missing M-lines. × 27000

to striated muscle resulting in an abnormal gene product in the cell membranes.

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THE EFFECT OF ANGIOTENSIN INFUSION, SODIUM LOADING AND SODIUM RESTRICTION ON THE RENAL AND CARDIAC ADRENERGIC NERVES

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Ljungqvist, A. The effect of angiotensin infusion, sodium loading and sodium restriction on the renal and cardiac adrenergic nerves. Acta path. microbiol. scand. Sect. A, 83 661–668, 1975

The renal and cardiac adrenergic nerv patterns in rats infused with large and small amounts of angiotensin nd in rats given NaCl plus DOCA, NaCl alone, and salt-free diets were examined by the histochemical fluorescence method. Infusion of small amounts of angiotensin led to a persistent blood pressure elevation whereas infusion of large amounts of angiotensin resulted in a transient rise in blood pressure, probably due to the development of tachyphylaxis. Nerve patterns were found to be normal in angiotensin-infused rats and in rats given NaCl. In rats given NaCl plus DOCA and in rats subjected to salt restriction, a partial or complete disappearance of the transmitter of the adrenergic nerve terminals were recorded. The findings suggest that angiotensin in itself is incapable of inducing visible alterations in the transmitter content of the terminals The findings agree with the view that angiotensin potentiates a norepinephrine depletion of the terminals during sympathetic activity since it can be assumed that increased plasma angiotensin levels as well as various degrees of increased sympathetic tonus were present in the rats subjected to salt restriction. The similar effect on the nerve terminals produced by the combined NaCl and DOCA administration is consistent with earlier reports of an increased turnover of norepinephrine in animals thus treated

Key words: Angiotensin infusion sodium renal nerves cardiac adrenergic nerves.

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It is known that an increased release of renin from the kidney can be induced by stimulation of the renal sympathetic nervous system (15 2) This may be the result of a direct adrenergic stimulation of the renin producing juxtaglomerular cells since these are surrounded by adrenergic nerve terminals (19)

In recent studies of renal hypertensive rats, a partial or complete disappearance of the transmitter content from the sympathetic nerve terminals was observed in the clipped kidney during the early phase of hypertension (13 12) Since the plasma angiotensin level is known to be elevated during the early phase of renovascular hypertension and since angiotensin is known to potentiate the release of noradrenalin from the terminals of stimulated sympathetic nerves (10 15) It was

suggested that the observed alterations were the result of an angiotensin effect on a hyperactive intrarenal sympathetic nervous system.

If this explanation holds true a primary activation of the sympathetic innervation of the juxtaglomerular cells would be involved in the development of renovascular hypertension. A possible, alternative explanation would be that the renin-angiotensin-aldosterone system was activated by extranervous mechanisms and that the consequent alterations in the intrarenal sodium/water balance exerted a direct effect on the nerve terminals.

The present investigation was undertaken with a view to assessing the influence of angiotensin and variations in sodium load on the juxtaglomerular adrenergic nerve terminals.

MATERIAL AND METHODS

Angiotensin Infusion

Twenty-four female Sprague-Dawley rats weighing between 180 and 200 g were used for this part of the study. The rats were anaesthetized by intraperitoneal administration of Nembutal. The right carotid artery was exposed and cannulated for continuous recording of the arterial blood pressure. The left carotid artery was exposed and cannulated for intra-arterial infusion of angiotensin (Hypertensin Ciba). Since highly different amounts of angiotensin were to be injected into the various rats at a constant rate of 0.15 ml of the solution per minute (see below) samples of angiotensin solutions of different concentrations were prepared. The rats were divided into three groups as follows

Group I — Seven rats into which between 50 and 75 µg of angiotensin was infused over 5–30 minutes.

Group II — Nine rats into which between 0.75 and 1.5 µg of angiotensin was infused over 10–15 minutes.

Group III — Eight control rats which were given an intra-arterial infusion of 0.15 ml/min of isotonic saline over a period of 10–15 minutes.

The kidneys of each rat were removed while the animal was still under infusion. With a view to comparison, the hearts were also removed and the organs processed as described below for examination of their adrenergic innervation patterns.

Salt Loading and Salt Restriction

Sixty female Sprague-Dawley rats were used in these experiments. At the beginning of the experiments, the weight of the rats ranged from 180 to 200 g. Blood pressure measurements were performed by the tail plethysmographic method. Before the experiments started, all rats had a blood pressure well below 140 mm Hg and were therefore regarded as normotensive. The rats were divided into the following groups

Group IV — Twenty rats in which DOCA-pellets were implanted intramuscularly. The rats were given a standard laboratory diet containing 0.4 per cent NaCl and a 2 per cent aqueous solution of NaCl *ad libitum* as drinking fluid.

Group V — Ten rats which were kept on the same food and drinking fluid as the rats of group IV but in which no DOCA-implantations were made.

Group VI — Twenty rats which were given the above laboratory diet from which the NaCl was withdrawn. Distilled water was given as drinking fluid.

Group VII — Ten control rats which were given the standard laboratory food and tap water.

Preliminary studies had suggested a certain mortality in rats given DOCA + salt and in rats subjected to salt restriction. Therefore these two groups of rats (groups IV and VI) were doubled when compared with the other groups (groups V and VII). In group IV however only two rats died during the experiments and only one in group VI.

The rats in groups IV–VII were followed by daily weight and blood pressure determinations for one week. Hypertension was considered to have developed when the blood pressure steadily exceeded 140 mm Hg, provided that this included a rise by 15 per cent, at a minimum of the initial level. On the 8th day the rats were anaesthetized by ether and the kidneys and hearts were removed for examination. The rats were then killed by an overdosage of ether.

All kidneys and hearts were examined with a view to their adrenergic innervation patterns, using the histochemical fluorescence method for demonstration of biogenic mono-amines (for references see 4, 9). For this purpose, pieces of tissue from each organ were rapidly frozen in liquid propane cooled by liquid nitrogen. This was followed by freeze-drying of the specimens at $-30°$ C upon which they were exposed to formaldehyde-gas for 1 hour at $+80°$ C (water content of formaldehyde-powder 0.6 per cent). Some pieces from each organ were processed directly as described, whereas other pieces were first incubated in α-methyl-norepinephrine of various concentrations ($5 \times 10^{-}$ M and 5×10 M) as previously described (16).

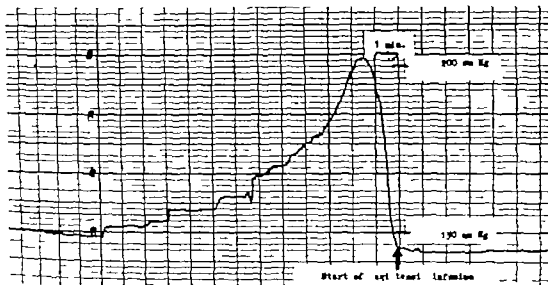


Fig 1 Recording of the blood pressure response to infusion of high doses of angiotensin into the rat (3.5 μg/min during 18 minutes) There is an early rise in blood pressure, followed by a slow return to normal levels. Cf. Fig. 2.

Subsequently all specimens were vacuum embedded in paraffin and 5–6 μ thick sections were cut and mounted in nonfluorescent medium (Entellan, Merck) to which xylol was added. The sections were examined in the fluorescence microscope. They were labelled according to a codified system and were examined without knowledge of grouping and clinical picture.

RESULTS

Angiotensin Infusion

As regards the control rats (group III) no effects on the blood pressure were recorded. In the rats infused with a highly concentrated angiotensin solution (group I) there was a rapid rise in blood pressure which, however gradually returned towards normal levels (Fig 1) In the rats infused with an angiotensin solution of low concentration (group II) there was a rapid rise in blood pressure which remained elevated until the animals were killed (Fig 2)

Examinations of the hearts and kidneys in the fluorescence microscope revealed no differences between the various groups of rats. In all kidneys, networks of yellowish-green fluorescent varicose fibres of the appearance typical of adrenergic nerve terminals were seen to accompany the arterial arborization up to the postglomerular capillaries (Fig 3B) No fibres were encountered in the glomeruli or along the veins. This picture is identical with the normal intrarenal adrenergic innervation pattern described previously (14 19)

In the hearts, an entirely normal innervation pattern as that described by *Winckler* (18) was also observed. Thus, fluorescent varicose fibres encircled the intramyocardial arteries and arterioles and were encountered in large numbers in the interstitial tissue between the muscle fibres (Fig 4B)

Any differences in nerve patterns in sections from pieces processed directly and from pieces incubated in α-methyl-norepinephrine before processing were not observed in any of the animals.

Salt Loading and Salt Restriction

In the rats subjected to salt restriction (group VI) and in the control rats (group VII) the same increase in weight was recorded during the experimental week (Table 1) and the blood pressures were normal. In the salt loaded animals, whether DOCA was given (group IV) or it was not given (group V) a significant decrease in body weight was recorded (Table 1) Blood pressure elevation

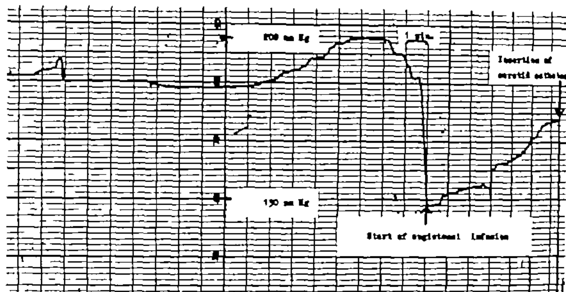


Fig 2 Recording of the blood pressure response to infusion of small doses of angiotensin (0.075 μg/min during 18 minutes) Insertion of the carotid artery catheter usually caused a rise in blood pressure and the infusion of angiotensin did not start until the blood pressure had normalized. The infusion resulted in an early rise in blood pressure which persisted at this higher level until the organs were removed. C.f. Fig 1

TABLE 1 *Body Weights and Blood Pressures at Start and End (1 Week Later) of the Experiments*

Group	No. of rats	Body weight		Blood pressure	
		Start	End	Start	End
IV	18	190 ± 5	155 ± 25	90 ± 5	175 ± 20
V	10	195 ± 5	125 ± 5	90 ± 5	80 ± 5
VI	19	200 ± 5	205 ± 10	90 ± 10	110 ± 15
VII	10	190 ± 5	200 ± 5	90 ± 10	100 ± 15

Rats in groups IV and V were salt loaded, those in group IV being supplemented with DOCA. Rats in group VI were given a salt free diet and distilled water and those in group VII were controls which had standard laboratory diets and tap water The figures are mean values ± SD

TABLE 2 *Fluorescence Reaction Indicative of the Presence of Adrenergic Transmitter Substance in the Nerve Terminals in the Heart and Kidneys of Rats Given NaCl and DOCA (Group IV), NaCl Alone (Group V), Salt-Free Diets (Group VI) and Normal Standard Diet (Group VII)*

Group	No of rat	Heart			Kidney		
		normal	reduced	absent	normal	reduced	absent
IV	18	0	3	15	0	10	8
V	10	10	0	0	8		0
VI	19	0	7	12	7	5	7
VII	10	10	0	0	10	0	0

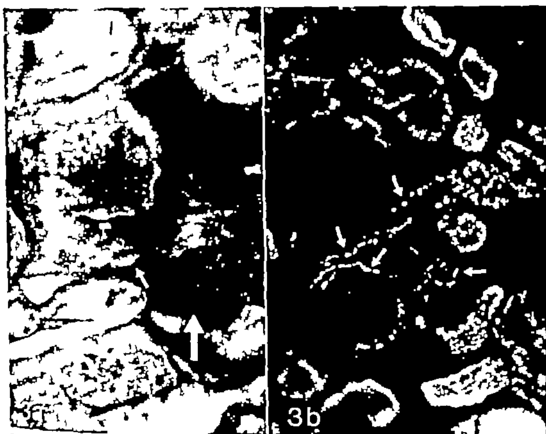


Fig. 3 A. Fluorescence microphotograph of the kidney of a rat that had been subjected to combined salt-DOCA treatment. An intrarenal artery (large arrow) and arteriole (small arrow) is seen. The latter leads to glomerular tuft. Autofluorescence of tubules no fluorescent varicose fibres are seen. × 350

Fig. 3 B. Fluorescence microphotograph of a section from the same kidney as that depicted in Fig. 3 A after incubation with α-methyl-norepinephrine. A normal nerve pattern with fluorescent varicose fibres around arteries and arterioles is seen (arrows). Autofluorescence of tubules. Dark area in centre, left corresponds to a glomerulus. × 350

occurred only in the rats given both salt and DOCA (group IV) but in these it was regular and often considerable (Table 1). This blood pressure elevation usually became evident as early as on the second day of the treatment, upon which it gradually increased.

The findings obtained by examination of the adrenergic innervation patterns of the hearts and kidneys in the various groups of rats are listed in Table 2. It can be seen that the control rats (group VII) displayed the above described normal patterns both in hearts and kidneys. In the experimental groups, no significant alterations were observed in the salt-loaded rats not given DOCA (group V): only in two of these rats did the non-incubated kidney specimens show a certain reduction in the density of the fibre networks along the intrarenal arterioles and this was associated with a comparatively weak fluorescence of the fibres along the finer intrarenal arterial branches. These nerve alterations are referred to as a "reduced" pattern. In the incubated specimens from these two kidneys, the nerve patterns were entirely normal.

Significant alterations of the intramyocardial and intrarenal adrenergic nerve patterns were observed in the non-incubated specimens from the salt loaded rats given

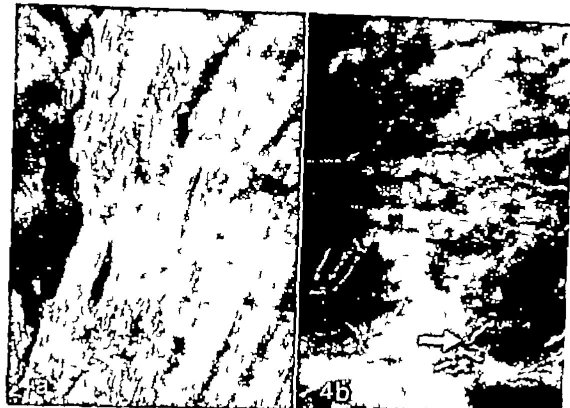


Fig 4 A Fluorescence microphotograph of the myocardium (left ventricle) of a rat that had been subjected to combined salt DOCA treatment. The dark area to the left corresponds to an artery branch No fluorescent varicose fibres are seen. × 350

Fig 4 B Fluorescence microphotograph of section from the same heart as in Fig. 4 A after incubation with α-methyl norepinephrine. A normal nerve pattern with fluorescent varicose fibres around an artery (arrow) and between the muscle bundles is seen. × 350

DOCA (group IV) and from the rats subjected to salt restriction (group VI) As regards the former rats, all hearts but one were completely devoid of fluorescent varicose fibres (Fig 3A) and in eight of these animals such fibres were also absent in the kidneys (Fig 4A) in 10 rats, the intrarenal nerve pattern was reduced. In the rats subjected to salt restriction, the nerve pattern was destroyed in twelve hearts and reduced in the remaining seven. As regards the kidney the corresponding figures were seven and five leaving seven kidneys with a normal nerve pattern.

As regards the incubated material, all sections of heart and kidney from rats in groups IV and VI showed normal nerve patterns (Figs. 3 B and 4 B)

DISCUSSION

The present investigation was initiated by the recent finding that, in rats made hypertensive by the production of unilateral renal artery stenosis, the early phase of the blood pressure elevation was associated with a disappearance of the adrenergic transmitter from the nerve terminals at the juxtaglomerular level in the stenosis kidney (13 1?) Since this alteration coincided with the period during which the plasma angiotensin level is known to be increasing and angiotensin is known to potentiate the release of noradrenalin from the terminals of stimulated sympathetic nerves (10 15) it was pointed out that the nerve alterations might reflect a sympathetic overactivity at the juxtaglomerular level which

would thus precede the development of hypertension and therefore possibly be of pathogenetic significance. An alternative theory would be that the renin-angiotensin-aldosterone system might be activated by extranervous mechanisms and that the consequent alterations in the intrarenal sodium/water balance would exert a direct effect on the adrenergic nerve terminals.

It was found in the present investigation that angiotensin alone is not capable of inducing visible alterations in the transmitter content of the nerve terminals, whether given in physiological pressor doses or in non physiological, large amounts. The failure of angiotensin to induce a sustained blood pressure elevation if infused in large amounts has been demonstrated by others (1) and has been ascribed to the development of tachyphylaxis (3)

Alterations in the nerve patterns were observed, however if salt loads of the animals were varied. These alterations were not restricted to the juxtaglomerular areas in the kidney but involved the entire organ, as well as the heart, suggesting that they were generalized.

It has previously been shown that NaCl + DOCA treatment of rats will lead to a decreased norepinephrine storage by the sympathetic nerves of various organs and that this occurs early during the treatment while the animals are still in the prehypertensive phase (5 6, 11) Later observations suggest that the sodium ion is the regulating factor and that the decreased norepinephrine storage is due to an increased turnover of the substance, indicating an increased release of norepinephrine from the nerves (7 8) These observations are in good agreement with the present findings of a partial or complete disappearance of fluorescent adrenergic nerve terminals in the heart and kidneys of rats given NaCl and DOCA.

de Champlin et al. (7) also found that administration of NaCl (or DOCA) alone was associated only with a minor decrease in the norepinephrine storage capacity This again is in agreement with the present findings of normal nerve patterns in the NaCl treated rats, but throws some doubt on the hypothesis that the sodium ion is of importance as a regulating factor in the peripheral turnover of norepinephrine Still it cannot be ruled out that in the absence of the sodium retaining hormone DOCA, the sodium load was simply too low to produce visible alterations.

In agreement with the hypothesis that sodium is capable of regulating the turnover of norepinephrine of the sympathetic nerves, *de Champlin et al.* (7) recorded an increased norepinephrine storage by the nerves during sodium restriction. This seems to contradict the findings in the present study where partial or complete disappearance of the adrenergic nerve terminals in the heart and kidneys was observed in many animals subjected to NaCl restriction. Animals thus treated, however are known to have elevated plasma renin and plasma angiotensin levels and angiotensin is known to potentiate the release of norepinephrine from the terminals of stimulated nerves (10 15) Any tendency of the sodium deficiency to induce an increased storage of norepinephrine by the nerve terminals is therefore counteracted by the elevated plasma angiotensin, provided that the sympathetic nerves are stimulated. Thus, whether one or the other factor will predominate in an animal depends probably upon the state of activity of the sympathetic nervous system in the animal concerned and, in this respect, individual variations are to be expected in laboratory animals subjected to experimental procedures. This would explain the variability in the appearance of the nerve patterns in the present animals subjected to NaCl restriction.

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Acta path. microbiol. scand. Sect. A, 83: 669–677, 1975

HISTOLOGY OF THE HUMAN CORPUS LUTEUM OF EARLY AND LATE PREGNANCY

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Visfeldt, J. & Starup, J. Histology of the human corpus luteum of early and late pregnancy. Acta path. microbiol. scand. Sect. A, 83: 669–677, 1975.

The aim of this investigation has been to examine which histological parameters are of value in the age determination of the corpus luteum of pregnancy. The material comprises representative ovarian biopsies from 16 women from the 10th through 42nd week of gestation. The following histological parameters have been found useful for the age determination of the corpus luteum: 1) the size of the granulosa cells; 2) the number of K-cells, vacuoles and colloid inclusions in the granulosa cell layer; 3) the size of the theca interna cells; 4) the number and size of vessels in the theca interna. Furthermore, the differentiation of the corpus luteum of pregnancy from the corpus luteum of menstruation is described, and some indications for a histological age determination of the corpus luteum of pregnancy are mentioned.

Key words: Corpus luteum of pregnancy; determination of age; histology.

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In a previous study we have shown that it is possible to date the corpus luteum of menstruation fairly accurately on the basis of histological parameters (7).

The aim of the present work has been to examine whether an age determination of the corpus lutum of pregnancy is possible on the basis of histological parameters. In this connection we also wanted to define the most characteristic differences between the corpus luteum of menstruation and that of pregnancy.

MATERIAL AND METHODS

The material consisted of corpus luteum biopsies from 16 pregnant women aged 15 to 39 years. It was a requirement that the women had been menstruating regularly prior to their pregnancy and that the date of the last menstruation was absolutely certain. The gestational age was calculated from the first day of the last menstruation. The subjects were divided into 2 groups. Group I comprised 8 women in the 10th to 23rd week of pregnancy and group II 8 women in the 36th to 42nd week. The women in group I were sterilized in connection with legal abortion, and in group II Caesarean section was carried out on various indications. Wedge-shaped biopsies including the entire corpus luteum were taken non-traumatically and submitted for histological examination.

The histological sections have routinely been

stained with haematoxylin-eosin and also by the method of van Gieson-Hansen. In some cases supplementary sections taken from the paraffin blocks have been prepared.

All sections have been evaluated under coat.

RESULTS

In most cases, it is easy to recognize the corpus luteum of pregnancy by inspection during operation. The size of the corpora lutea of pregnancy varies greatly some of them having fairly large cystic, central cavities. Generally we have found that the corpora lutea from the first period of pregnancy are larger than those from the last period. We have not measured the corpora lutea because, in our opinion, differences in their diameter due to varying size of the central cavity might greatly influence the results. Resection of ovaries during the early stages of pregnancy results in more bleeding than late in pregnancy

Histological Parameters

A number of histological parameters were evaluated semiquantitatively as will be seen from Table 1 The following grading was applied the presence of a central cavity + — the thickness of the internal fibrous layer the granulosa cell layer and the theca interna layer — + ++ +++ the size of the granulosa cells and the theca cells + ++ +++ the number of K-cells, inclusions and vessels — + ++ +++

The Central Cavity

As appears on macroscopical inspection, the central cavity varies greatly in size but, on an average, it is largest in the early pregnancy group. Only one corpus luteum (from the 42nd week) had no central cavity The cavity is filled with fluid or with a coagulum infiltrated by a greater or smaller number of fibroblasts. In the peripheral part the proliferation of fibroblasts is often particularly pronounced, collagen fibrils are formed, and a grad al transition into a fibrous layer covering the nulosa lutein layer may occur

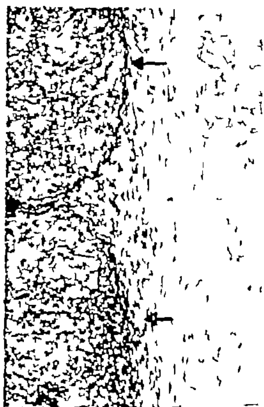


Fig 1 10th week of pregnancy Marked variation in the inner fibrous layer within the same corpus luteum. H–E × 87

The Internal Fibrous Layer

The thickness of this layer varies greatly from one corpus luteum to another but also within the same corpus luteum (Fig 1) If only for this reason, the thickness of the layer is not well suited for semiquantitative evaluation. We were unable to demonstrate any definite difference in the thickness of the layer between early and late corpora lutea

The Granulosa Lutein Layer

The undulating granulosa cell layer is the largest tissue component seen in all corpora lutea. The undulations are less pronounced in the rather cystic corpora lutea On the whole the layer is thickest in the early corpora lutea.

Two characteristic types of cell are present (Fig 2 a + b) The typical granulosa cell is round or polygonal rather large with a fine granular light and slightly eosinophilic cyto-

TABLE 1 *Useful Histological Parameters of The Corpus Luteum in Early and Late Pregnancy*

Age (years)	Gestational age (weeks)	Central cavity	Granulosa-lutein layer			Inclusions		Theca interna		Vessels
			Thickness	Size of cells	K-cells	Colloid	Vacuoles	Thickness	Size of cells	
26	10	+	++	++	+++	—	++	+++	+++	+++
31	10	+	++	+++	+++	+	+++	+++	+++	+++
33	10	+	++	++	++	+	+++	++	+++	++
30	11	+	+++	++	+++	++	++	++	+++	++
37	14	+	+++	++	+	+	++	++	++	++
15	18	+	++	++	+	++	++	++	++	+
36	19	+	++	++	+	+	++	++	++	++
31	23	+	++	++	+	++	+	++	++	++
24	36	+	++	++	—	++	+	++	+	+
38	38	+	++	+	—	++	+	++	+	+
34	39	+	++	+	—	++	+	++	+	+
27	40	+	++	+	—	+	++	++	+	+
31	40	+	++	+	—	+	+	+	+	+
34	40	+	+	++	—	+	++	+	+	+
39	41	+	++	+	—	+	+	+	+	+
24	42	—	+	+	—	++	+	+	+	+



2a
b
3a
b

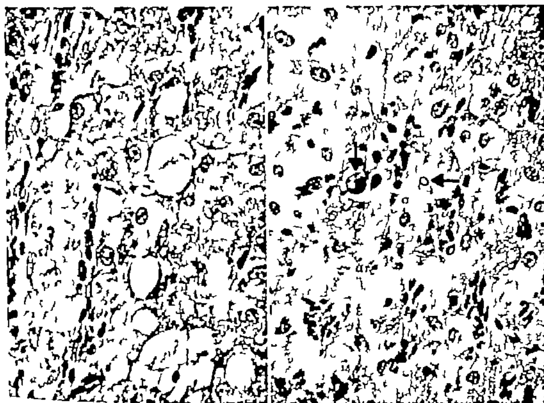


Fig. 4 10th week of pregnancy Numerous vacuoles of varying size. H–E × 350.

Fig. 5. 38th week of pregnancy Colloid inclusions (arrows) and few vacuoles. H–E × 350

plasm, often with poorly defined cell membranes. The nucleus is large and round with a regular fine chromatine structure and a distinct nucleolus. The cells and the nuclei are largest in the early corpora lutea.

The other type of cell is characterized by a heavily eosinophilic cytoplasm, a smaller hyperchromatic nucleus and often a stellate shape. This type of cell is characteristic for early corpora lutea and, in our material, the largest number of these cells was found during the 10th to 11th week (Table 1). They were rarely observed in the late corpora lutea (Fig 3 a + b). The presence of these cells is very important when distinguishing between early and late corpora lutea. The cells are generally designated "dark cells" or "K-cells". Intermediary forms between the K-cells and the other granulosa lutein cells may appear.

Fig. 2 a + b 10th week of pregnancy Granulosa-lutein layer with large pale granulosa cells and dark stellate K-cells. H E; × 140 (a) × 350 (b)

Fig. 3 a + b 39th week of pregnancy Granulosa-lutein layer with smaller granulosa cells and no K-cells. H–E × 140 (a) × 350 (b)

Cell Inclusions

Two types of inclusion are listed in the Table, i.e. vacuoles and colloid. Vacuolized cytoplasm was found in the granulosa cells from all corpora lutea perhaps to a somewhat larger extent in the early than in the late stages (Fig 4). Large colloid inclusions were very typical and were present most frequently in the late corpora lutea (Fig 5). However all transitory forms seemed to be present, from colloid droplets to quite small inclusions, almost resembling secretory granules.


6a
b
7a
b



Fig. 8. 10th week of pregnancy. Large vessels in theca interna and adjacent ovarian stroma. H–E × 56.

Fig. 9. 39th week of pregnancy. Small vessels in theca interna and adjacent ovarian stroma. H–E; × 56.

Theca Interna

The theca interna layer surrounds the granulosa cell layer. This layer varies in thickness and protrudes as triangular trabeculae into the granulosa cell layer. Both at the border and in the trabeculae the theca cells surround larger and smaller vessels.

Generally the layer is thickest in the early corpora lutea, and the morphology of the cells varies during pregnancy. In early corpora lutea the cells are round or polygonal with well defined cellular borders, and with a granular cytoplasm which is somewhat darker than that of the granulosa cells. The nucleus is round and situated centrally with a distinct nucleolus (Fig 6 a + b). In the late corpora lutea, a smaller number of cells is found both around the granulosa cell layer and in the trabeculae, and the character of the cells changes. The cells are smaller with less cytoplasm and the borders are not so well defined. The nuclei are darker, more irregular and, in some cases, rather oblong (Fig 7 a + b), so that the cells might resemble fibroblasts.

Apart from the typical theca cells, there are, in the early corpora lutea, also a few cells which morphologically and also as regards stainability resemble very much the K-cells mentioned in the description of the granulosa cell layer. They were less abundant than in the granulosa cell layer and their occurrence follows that of the K-cells in the granulosa cell layer.

Fig. 6 a + b. 10th week of pregnancy. Theca interna layer with rather large uniform, [illegible] defined theca cells. H.-E; × 140 (a), × 350 (b).

Fig. 7 a + b. 39th week of pregnancy. S[illegible] varying theca cells with darker [illegible] H.-E; × 140 (a), × 350 (b).

Theca Externa

Generally a characteristic theca externa layer is not visible. The adjacent ovarian stromal tissue is abundantly vascularized, but in other respects not histologically well defined. Luteinized stromal cells are often demonstrable, but it remains to be clarified whether these cells are developed around the corpora lutea or whether they originate from adjacent follicles, remnants of corpus luteum of menstruation or atretic follicles, around which a layer of luteinized stromal cells is found in ovaries from pregnant women (6).

Because the theca externa layer could not be characterized as a well defined histological parameter the evaluation is not shown in Table 1.

Vessels

From the theca externa, vessels extend into the theca interna layer where they are visible in varying sizes surrounded by connective tissue. In the early corpora lutea many often large, vessels are present in the theca interna, most distinctive at the base of the trabeculae (Fig 8). The vessels pass centripetally into the trabeculae and in between or around groups of granulosa cells, mostly as thin vessels or capillaries. The late corpora lutea contain considerably fewer and smaller vessels (Fig 9) and many obliterated vessels are seen. The amount of connective tissue around the vessels increases in proportion to the decreasing vascularization and regression of the theca interna cells.

DISCUSSION

The very rapid physiological changes in the normal menstrual cycle reflect themselves, as previously described (1 2 3) in quite characteristic histological changes in the corpus luteum of menstruation. A number of histological parameters can form the basis of a more accurate dating of the corpus luteum during the menstrual cycle (7). The changes in the corpus luteum of pregnancy are much less pronounced, and an attempt to evaluate these histological parameters quantitatively has shown that, first and foremost, only slight differences in degree exist.

Our material is modest, but it will be extremely difficult to increase group I, since sterilization today is mostly done by means of laparoscopy in which case it is not possible to obtain representative biopsies of the ovaries. However the material is sufficiently comprehensive to allow the following conclusion to be drawn. The histological parameters characteristic for early corpora lutea are 1) presence of many K-cells in the granulosa cell layer 2) large size of the granulosa cells, 3) the many rather large theca lutein cells, and 4) the many and often large vessels. On the other hand, the late corpora lutea are characterized by 1) few or no K-cells, 2) smaller granulosa cells, 3) a thinner theca cell layer with small and rather dissimilar cells, 4) more connective tissue and less vascularization and, in particular 5) occurrence of large and small colloid inclusions.

We have not been able to find any essential difference in the thickness of the granulosa cell layer in early and late corpora lutea. The central cavity shows considerable variations from one corpus luteum to another and this applies also to the internal fibrous layer even in the same corpus luteum. Consequently these parameters do not appear to be of value in the differentiation between early and late corpora lutea.

Our findings show good agreement with those described previously by e.g., *Nelson et al.* (5) and *Karjalainen* (4). Both these publications include comprehensive bibliographies, to which reference is made.

The Value of Histological Examination of Corpus Luteum in Pregnancy

Histological evaluation of the corpora lutea may be indicated in certain forensic cases, or when an evaluation of the effect of different hormone preparations on the function of corpus luteum is required.

A thorough knowledge of the histology of the corpus luteum, in both early and late stages of pregnancy is a prerequisite for

distinguishing between the corpus luteum of pregnancy and that of menstruation. As a rule, this differentiation is rather easy but may in exceptional cases present some difficulties, in that, on the 8th to 10th day after ovulation, the corpus luteum of menstruation may sometimes resemble the corpus luteum of pregnancy Amongst the most important differential diagnostic parameters are the K-cells in early corpora lutea of pregnancy and colloid droplets in the late stages.

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Acta path. microbiol. scand. Sect. A 83 678–682 1975

SKELETAL FLUOROSIS IN IMMOBILIZED EXTREMITIES

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Rosenquist, J B Skeletal fluorosis in immobilized extremities. Acta path. microbiol. scand. Sect. A, 83 678–682, 1975

The effect of immobilization on skeletal fluorosis was studied in growing rabbits. One hind leg was immobilized by an external fixation device extending below the wrist joint and above the knee joint, the extremity being in a straight position after severance of the sciatic nerve The animals, aged 7 weeks at the beginning of the experiment, were given 10 mg of fluoride per kg body weight and day during 12 weeks. In the tibiae, development of the skeletal fluorosis was more irregular than that observed in previous studies of normally active animals, being most excessive in the mobile bone. The immobilization effect was most profound in the femora as the cortical thickness and the femur score were significantly higher than those in the mobile femora. It was suggested that an altered muscular activity was the reason for the observed changes.

Key words Fluorosis, skeletal immobilized extremities.

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Prolonged intake of high amounts of fluoride might give rise to skeletal fluorosis which is characterized both by excessive, irregular bone formation and increased bone resorption (cf *Singh & Jolly* 1970) In a previous experiment on growing rabbits, excessive fluorotic changes localized to the lateral aspect of the middiaphysis of the tibia developed. In contrast, the femur remained almost unaltered in spite of similar fluoride concentrations in the two bones (*Rosenquist* 1973 a and b) Thus, the development of bone fluorosis was suspected to be influenced by some additional factors. Differences in loading of the two bones could be one such factor The present investigation was therefore undertaken with the aim of studying whether inactivation of the hind leg of growing rabbits would change the pattern of fluorotic alterations in the tibia and femur

MATERIAL AND METHODS

Nine growing rabbits of both sexes, 7 weeks old at the beginning of the experiment, were used for the study Under anaesthesia produced by intravenous application of sodium pentobarbital, the sciatic nerve of one hind leg was severed in order to produce partial paralysis. The leg was then padded with a strip of polyvinyl and immobilized by an external fixation device extending below the wrist joint and above the knee joint, the extremity being in straight position The animals were given 10 mg of fluoride per kg body-weight per day during 12 weeks.

After 12 weeks, the animals were killed by an overdose of sodium pentobarbital. The femora and tibiae were freed from soft tissues. Within each pair of femur and tibiae, the bones were compared with regard to the extent of the morphological changes. The examinator did not know which bones had been immobilized. By dividing the bones at right angles to their long axis, one sample was obtained from each middiaphysis. In the tibiae, the area distal to the fibulotibial synostosis was chosen. A second sample from the tibia was obtained 5 mm proximal to the distal epiphyseal zone. Great care

is taken to ensure that the samples were from identical parts of the respective bones. A small proximal part of each sample was defatted and dehydrated in acetone which was changed daily for three days and the sample was used for fluoride determinations. The other bone segments were dehydrated in absolute ethanol and embedded in methyl methacrylate. Cross sections were sawn from the proximal end and ground down to a final thickness of 80–100 µ.

Microradiography was performed as described earlier (*Rosenquist* 1973 b) the sections being in direct contact with Kodak spectroscopic plates 649-0.

Bone thickness of the femur was determined on the microradiographs by measuring the largest external (ED) and internal (ID) diameters in medio-lateral direction and ventro-dorsal direction in right angles to each other (Fig. 1) The measurements were done by means of an eyepiece micrometer (Zeiss) at × 20 magnification. The average cortical thickness was calculated by dividing with 4 the sum of the ventral dorsal lateral and medial parts of the cortex. The relative cortical thickness (femur score) was expressed as the sum of the cortices (ED minus ID) in each direction in per cent of ED

Owing to the irregular pattern of fluorosis in the tibia, measurements were not done on specimens from that bone.

Fluoride Determination

The acetone treated bone samples for fluoride determination were air dried The tibial circum-



Fig 1 Microradiograph of the cross section of control femur of normal morphological appearance. The cortical thickness was determined by measuring the largest external (ED) and internal (ID) diameters in two directions at right angles to each other The average thickness was calculated by dividing with 4 the sum of the differences (ED minus ID) in the two directions Original magnification × 9.



Fig 2 Microradiograph of the cross section of the experimental femur which presented the most pronounced morphological changes. The width of the ventral (V) part of the cortex is increased due to extensive periosteal bone formation. In its superficial parts, this is trabecular without formation of osteones. A large number of resorption cavities are seen within the cortex. Original magnification × 9

ferences were divided into lateral and medial parts which were pulverized separately in a Wiley Laboratory Mill (Intermediate Model) provided with a 40 mesh sieve. The femora were divided into a ventral and a dorsal piece and pulverized as described above. The bone powder was ashed at 700° overnight The fluoride determination was done on about 10 mg of ash as described previously (*Rosenquist* 1973 a) using a fluoride electrode (*Frant & Ross jr* 1966)

Statistic

The degree of significance was tested by means of Student t-test.

RESULTS

Due to wound infection, one animal was killed during the observation period. All other 8 remained healthy No pressure wounds had developed during the observation time.

The inactivated leg will hence forward be called experimental, the not inactivated one being termed the control.

Gross Morphological Examination

Thirteen femora were of normal appearance without signs of exostosis formation. In 3 cases, the diaphysis of the experimental femur was covered on its ventral and distal parts by a periosteum that was thicker

that on its control pair. The underlying bone was rougher, thicker and of somewhat lighter colour than normal bone.

Exostoses had developed in 7 out of 8 control tibiae and in 4 out of 8 experimental bones. The exostoses were in half of the specimens localized to the lateral part of the tibia, usually distal to, and at varying distances from the fibulotibial synostosis. In one experimental bone and in 3 control bones, the exostotic changes were seen at the medial aspect of the tibiae.

Microscopic Examination

Femur. All experimental femora showed considerable periosteal bone formation at the lateral and medial or ventral aspects of the cortex. This bone formation was accompanied by resorption in adjacent areas in 7 out of 8 animals. Resorption always occurred intracortically and in 5 samples, endosteal resorption was also noted. The net result of the bone changes was a thickened femoral cortex.

In three control femora changes similar to those described above including endosteal resorption were seen. However a comparison between each femur pair revealed more excessive bone changes in 7 out of 8 experimental femora. In the eighth pair the bones did not differ morphologically.

Tibiae. In the specimens from the *distal* part of the tibia, exostotic trabecular bone formation was seen in four experimental tibiae and in four control tibiae. Only in one case, both tibiae showed such bone formation. In all these bones, intracortical bone resorption was seen, the resorption cavities even being within the exostotic trabecular bone. Furthermore, endosteal bone resorption was seen in three tibiae, one of which showed no signs of trabecular bone formation.

In the midtibia, *i.e.* the area immediately distal to the fibulotibial synostosis, the exostotic bone formation was more excessive than in the distal part of the tibia. The changes were seen at the lateral and/or the medial part of the circumference. Although the differences between each pair of tibiae were slight, they were found to be more pronounced in the specimens of the control tibiae than in their experimental pair in seven animals. In the eighth rabbit, the opposite was true.

TABLE 1. *The Average Thickness of the Femur Cortex and the Femur Score of Control and Experimental Bones M ± S.D*

	Control n = 8	Experimental n = 8
Average cortical thickness mm	1.09 ± 0.03***	1.32 ± 0.11
Femur score	31.2 ± 0.7**	33.8 ± 1.8

** $0.001 < p < 0.01$
*** $p < 0.001$

Cortical Thickness of the Femur

The cortices of the experimental femora were significantly thicker ($0.001 > p$) than those of the controls (Table 1). Moreover the femur score was significantly higher in the experimental bones ($0.01 > p > 0.001$) than in the controls.

Fluoride Concentration

The fluoride concentration was the same in the two parts of each *femur* analysed (Table 2) and with regard to treatment, there was no difference between the femora.

The fluoride concentrations varied in the

TABLE 2. *Fluoride Content in Per Cent of Ash in the Different Parts of Femur and Tibia of the Control and Experimental Bones. M ± S.D*

	Control n = 8	Experimental n = 8
Femur –		
ventral	0.57 ± 0.09	0.62 ± 0.11
dorsal	0.67 ± 0.09	0.71 ± 0.06
Distal tibia –		
medial	0.67 ± 0.15	0.70 ± 0.10
lateral	0.64 ± 0.15	0.68 ± 0.10
Midtibia –		
medial	0.70 ± 0.17	0.55 ± 0.13
lateral	0.55 ± 0.11	0.63 ± 0.07

* $0.05 > p > 0.02$

different parts of the *tibia* (Table 2) They were slightly higher in samples from the medial parts of the control tibiae ($0.05 > p > 0.02$) than in samples from the lateral parts. A comparison between areas with signs of bone formation, observed at microscopy and adjacent "normal" areas showed fluoride concentrations of the same magnitude.

DISCUSSION

In previous studies (*Rosenquist* 1973 a and b) growing rabbits of the same age as those used in the present investigation were given the same amount of fluoride in the same way during almost the same length of time (14 weeks instead of 12 weeks) The average fluoride concentrations were of the same magnitude, 0.75 per cent F instead of 0 62 per cent found here. A consistent finding in the previous study was the localization of fluorotic exostoses to the lateral aspect of the tibial middiaphysis and the femoral changes were restricted to minor signs of increased bone activity within the ventral part of the diaphyseal cortex (*Rosenquist* 1973 b)

Inactivation of one hind leg markedly changed the picture of bone fluorosis This alteration was most profound in the experimental femora where periosteal bone formation was obvious, which is in contrast to findings in the previous study namely almost lack of periosteal bone formation in the mobile femur (*Rosenquist* 1973 b)

The manifestations of skeletal fluorosis were only slightly different in control and experimental tibiae. However they were found to be more advanced in the control bones. In the present study the fluorotic exostoses were found in different areas of the tibia and did not follow the previously observed pattern (*Rosenquist* 1973 b)

The fact that the fluoride concentrations were similar in all femora and tibiae, "inactivated" or not, indicate that the "fluoride pressure" on the bone cells was the same in the bones studied. Thus, the morphological difference between the normal and experimental bones was not due to differences in fluoride exposure.

In the experimental model used, it was not possible to distinguish between the single effect of unloading immobilization and denervation respectively However the experimental femur as well as the experimental tibia became less than normally loaded. It therefore seems unlikely that a decrease in loading would diminish the morphological changes in the tibia while they were increased in the femur

The degree of immobilization of the experimental femur and tibia differed the femur being most mobile. However the mobility of the experimental femur was decreased as compared with its pair control or with femora of fluoride fed rabbits with normal mobility (*Rosenquist* 1973 b) In these, only minor changes in bone activity were seen which could be attributable to fluoride ingestion. Moreover as the immobilization was most complete in the tibia where the signs of fluorosis were less marked it does not seem likely that the less decreased mobility of the femur would explain the change in morphology

Severance of the sciatic nerve affects all tibial and fibular muscles whereas only the hamstrings of the femur are denervated. By innervation of the adductor muscles, the knee joint extensors and the flexor muscles of the hip joint remain intact. The resulting change in the pattern of muscular activity could explain the increased periosteal bone formation.

Disuse osteoporosis known to appear in inactivated limbs (cf *Geiser & Trueta* 1958) did apparently not develop Instead, the cortex of the experimental femur was thicker than that of the control bone and the femur score was higher However it must be borne in mind that these observations fail to give sufficient information about the skeletal mass which otherwise might elucidate the question of disuse osteopenia.

The present investigation has shown that the extent and pattern of bone fluorosis is not only determined by the fluoride ingest also by other factors of which

muscular pattern seems to be the most important.

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EXPERIMENTAL MURINE LEPROSY

6 *Cellular Reactions in the Draining Lymph Node after Injection of* Mycobacterium lepraemurium *into the Foot Pads of Mice*

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Haugen, O. A. & Closs, O. Experimental murine leprosy 6. Cellular reactions in the draining lymph node after injection of *Mycobacterium lepraemurium* into the foot-pads of mice. Acta path. microbiol. scand. Sect. A, 83 683-692, 1975.

The reaction patterns of the draining lymph nodes were studied in C3H and C57/BL mice after foot-pad inoculation of *Mycobacterium lepraemurium* (MLM). Bacilli were found in the lymph nodes of both strains already a few days after inoculation, but any marked reaction did not occur until approximately 3 weeks later and then only in the lymph nodes of C57/BL mice. The reaction involved enlargement, epithelioid cell granuloma formation and proliferation of pyroninophilic blast cells in the T-cell area. The lymph nodes in these animals remained large and showed reactive pattern for up to 230 days. At this stage, bacilli were relatively few. In C3H mice, no reactive changes developed and it was not until much later that they became significantly enlarged. In the advanced stage, normal lymph node structures were replaced completely by giant macrophages loaded with acid fast bacilli. C3H and C57/BL mice are polar as regards the lymph node reaction against MLM. In C3H mice it resembles the advanced stages of human leprosy while it in C57/BL mice mimics the situation in the BT TT region. Immunofluorescence studies did not reveal any early proliferation of Ig-containing cells and the lack of cellular immune reaction against MLM in C3H mice cannot be explained as an early B-cell reaction to interfere with the development of cell mediated immunity.

Key words: Leprosy murine lymph nodes cellular reaction.

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Murine leprosy seems to exhibit an immunological and histopathological spectrum which resembles that of human leprosy (*Closs & Haugen* 1974). In response to a local injection of *Mycobacterium lepraemurium* (MLM) C57/BL mice develop a granulomatous reaction which prevents a further spread of the infection. In C3H mice, on the other hand, the infection proceeds without any detectable host reaction (*Closs & Haugen* 1975 a, b). Hence the types of MLM-infection to occur in these strains seem to represent polar forms of murine leprosy.

Humoral antibodies may suppress the expression of cell mediated immunity (*Parish* 1972, *Waksman* 1973) and the lack of cellular immune reaction against MLM in C3H mice might be assumed to be due to an early B-cell response leading to the production of humoral antibodies which in turn inhibit the T-cell function. Accordingly it is of importance to have the early reaction to MLM infection in the two strains C57/BL and

characterized with regard to T and B lymphocyte responses. In the present investigation this problem was approached by studying the cellular reaction in the popliteal lymph node after injection of MLM into the foot pad.

MATERIALS AND METHODS

Experimental animals. Specifically pathogen free (spf) female mice of the inbred strains C3H/A and C57/BL/6J were obtained from Gl. Bomholt gård Ltd., Denmark. The animals weighed 16–18 grams at the beginning of the experiments; they were fed pellets (Norwegian Standard Stock No. 1 Mice and Rats) and were allowed tap water *ad libitum*.

Propagation of bacilli. *Mycobacterium lepraemurium* (MLM) Douglas strain was propagated in the outbred strain NMRI and harvested as previously described (*Closs & Haugen* 1974).

Inoculation procedures. 10 microlitres of a bacillary suspension containing 2.7×10^{8} bacilli per ml were injected superficially into each of the hind foot pads. To prevent leakage of the inoculum, the injection site was sealed by plastic spray (Nobecutan "Bofors").

Histological examination. At certain intervals of time, four animals of each strain were killed. Tissues including foot pads, the popliteal lymph nodes, the inguinal lymph nodes, spleen, liver and thymus were removed, fixed and embedded as previously described (*Closs & Haugen* 1974). Sections were stained with haematoxylin and eosin (HE) and, for the demonstration of acid-fast bacilli, by a modified Ziehl-Neelsen technique (*Putt* 1951) and by auramin rhodamine. Sections from all lymph nodes were also stained with methyl green pyronine (*Trevan & Sharrock* 1951).

Planimetry of lymph nodes. Using an ordinary slide projector, the microscopic sections were enlarged and the outer aspects of the lymph nodes were drawn on paper. For this purpose, the largest of the appearing sections from each case was selected. The size (in arbitrary units) of each lymph node was measured by a standard planimetric technique.

Production of rabbit anti mouse immunoglobulin antiserum. Three ml of normal mouse serum was mixed with 2 ml of packed, washed *Staphylococcus aureus*, strain Cowan I, to absorb the immunoglobulin onto the staphylococci (*Kronwall* 1971). The mixture was centrifuged at 2000 × g for 20 min, the supernatant was discarded and the pellet washed × 6 with physiological saline containing 0.01 M phosphate buffer pH 7.5 (PBS). Finally the bacteria were resuspended to make a 10 per cent suspension which was frozen immediately and stored at −20° C until used. Three rabbits were injected intramuscularly with 0.5 ml of the staphylococcal suspension into each thigh and the injections were repeated at days 14, 24, 34 and 61. Nine days after the last injection the rabbits were bled from the ear vein and the serum fraction was collected and stored at −20° C. The sera were tested in immunoelectrophoresis against normal mouse serum. The best antiserum which produced one strong precipitation line in the gamma region and a faint line in the alpha region, was used for preparation of immunoglobulin and labelling with fluorochrome without further absorption.

Fractionation of antiserum. The immunoglobulin fraction of the antiserum was isolated by the method described by *Brandtzaeg* (1973 a). Twenty ml of antiserum was treated with DEAE Sephadex A50 at pH 8.0 to remove most of the unwanted proteins and then precipitated with ammonium sulphate at half saturation, i.e. 1.95 M, and left overnight at 4° C. The precipitate was dissolved in distilled water and dialysed against PBS containing 0.01 per cent merthiolate. The protein concentration was determined spectrophotometrically at 280 nm ($E^{1\%}_{1\,cm} = 14$) and found to be 33 mg/ml.

Labelling of immunoglobulins. The method of *Brandtzaeg* (1973 a) was used. Two ml of the mouse immunoglobulin solution was mixed with 4 ml of PBS at room temperature. Forty micrograms of Tetramethylrhodamine Isothiocyanate (MRITC) (Baltimore Biological Laboratories, Baltimore, Md. USA) was added per mg protein under continuous stirring. The pH was adjusted to 9.50 with 0.1 N NaOH and kept within ± 0.05 pH units throughout the conjugation. After 1 hour, unreacted fluorochrome was removed by passage through a Sephadex G-50 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The first peak containing the labelled immunoglobulins was then fractionated by anionic-exchange chromatography on a Whatman DE52 column (W. & R. Balston, Ltd., England) previously adjusted to pH 7.6 with 0.5 M NaH_2PO_4 and equilibrated with 0.01 M phosphate buffer pH 7.6. The fall-through fraction was collected and used in the present study. The light absorption of the conjugate was measured at 280 nm and at 515 nm and the OD ratio was found to be 6.0. Finally the conjugate was dialysed against PBS containing 0.01 per cent NaN_3.

Immunofluorescence of lymph nodes. Excised specimens of popliteal lymph nodes were washed in PBS for 24 hours at 4° C, fixed in ethanol and embedded according to *Sainte-Marie* (1962). Deparaffinized sections were pre-incubated for 30 min with 20 per cent bovine serum albumin, washed, and thereafter incubated for 30 min at room temperature with the conjugate diluted 1:4 in 20 per cent bovine serum albumin (*Brandtzaeg* 1973 b).

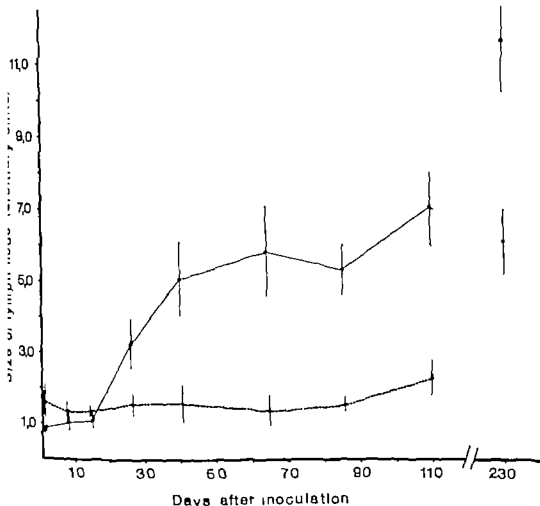


Fig. 1. Diagram showing changes in the size (in arbitrary units, mean ± SEM) of the popliteal lymph nodes in C3H mice (broken line) and C57/BL mice (unbroken line) at various intervals after foot-pad inoculation of *M. lepraemurium*.

The preparations were then washed with PBS for 3 × 15 min; washed briefly in distilled water and mounted in polyvinyl alcohol (Elvanol-DuPont de Nemours & Co., Wilmington, Del. USA) dissolved in glycerol and PBS in the ratio 1 : 4 (W/V). Microscopy was performed using a Leitz Orthoplan microscope equipped with an Opak illuminator for incident light. Photographs were taken with an Orthomat automatic camera, using Kodak Plus-X film.

RESULTS

Acid-fast bacilli were occasionally found in the marginal sinus of the popliteal lymph node 48 hours after inoculation. Thirteen days later bacilli could be demonstrated at this site in nearly all animals, but even at this stage bacilli were scanty and could be seen only in the auramin rhodamin stained sections.

The lymph node of the popliteal region was hardly visible to the naked eye and appeared as a rounded, greywhite nodule with a translucent capsule in the dissection microscope. During the first two weeks following inoculation there was no change in the size of the popliteal lymph nodes in either of the strains (Fig. 1). The histological appearance of the lymph node was apparently identical

in the two strains 2, 8 and 14 days after inoculation. Secondary follicles with germinal centres were not prominent and in both strains, scattered, large pyroninophilic cells could be seen in the paracortical area. Histiocytes were often numerous in the marginal sinuses and in the medullary region, but plasma cells were rare and occurred mostly as single cells. At this stage small and medium sized cells of the appearances of small lymphocytes were the most frequent cell type encountered (Fig 2 A)

Four weeks after inoculation, a marked enlargement of the popliteal lymph nodes occurred in all C57/BL animals (Fig 1) The nodes were clearly visible to the naked eye and showed marked hyperaemia of the capsular vessels and capsular distension. The lymph nodes in C3H mice remained small and pale. Histologically the paracortical areas of the popliteal lymph nodes in C57/BL mice contained a large number of pyroninophilic blast cells (Fig 2 B) These cells were usually scattered over a wide area. Mitotic figures were also seen at this stage and occasionally blast cells also appeared within the lumina of lymph sinuses which were wide and contained several small lymphocytes. Small eosinophilic nodules consisting of large, irregularly shaped cells with a vesicular nucleus reminding of epithelioid cells had also started to form throughout the lymph nodes. Bacilli were found in all animals at this stage and appeared to be more numerous than after 14 days. The bacillary content of the lymph nodes was, however approximately the same in both strains. The popliteal lymph nodes of C3H mice showed no proliferation of blast cells and the morphology of the lymph nodes appeared largely unchanged.

During the next weeks, a further increase in size of the popliteal lymph nodes occurred in C57/BL animals (Fig 1) Histological examination 41 days after inoculation revealed that the number of more typical epithelioid cell infiltrates (Fig 2 C) was markedly increased they were distributed in a multicentric fashion, leaving the cortical zone free There was still a marked dilatation of lymphatic sinuses, but the number of lymphocytes in their lumina was apparently lower than that at four weeks. Pyroninophilic blast cells were still found in the paracortical area, but were less numerous. Small lymphocytes were the most prominent cell type and the number of plasma cells was not increased. Secondary follicles with a prominent germinal centre showing cells in mitosis and blast-like cells were occasionally seen. In auramin rhodamin stained sections, bacilli were found within epithelioid cell nodules, but the number of fluorescent rods was lower than that after four weeks. The lymph nodes of C3H mice showed no enlargement, no blast cell proliferation and no epithelioid cell infiltrates.

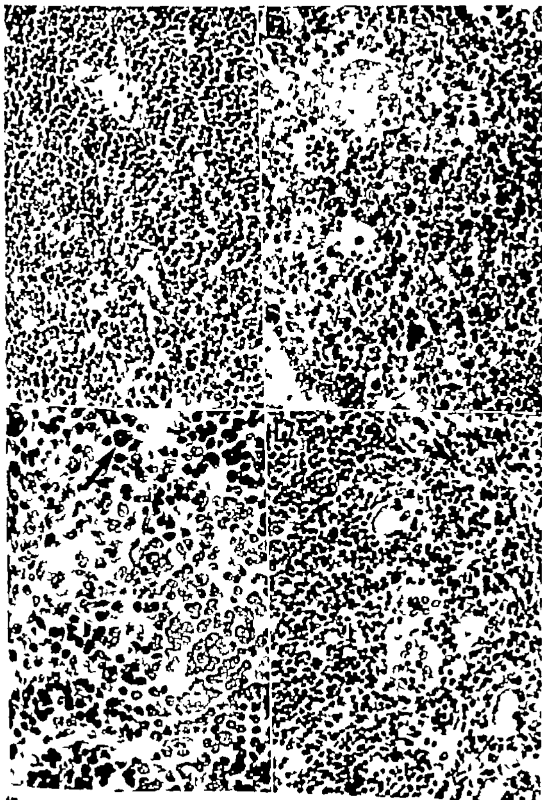
The subsequent development of the changes observed in C57/BL mice can be summarized as follows the popliteal lymph nodes remained large throughout the observation period covering up to 230 days (Fig. 1) Coalescing and multicentric epithelioid cell infiltrates persisted until termination of the experiment. By day 65 and later an increasing number of pyroninophilic cells with abundant cytoplasm and small nuclei were seen close to lymphatic sinuses and in the vicinity of epithelioid cell infiltrates. Occasionally large, pyroninophilic blast cells were also seen in the paracortex. The number of bacilli in the regional lymph node remained low being less than 10 per section In some of the ani-

Fig 2 A Paracortical area of the popliteal lymph node of a C3H mouse 48 hours after foot-pad inoculation of *M lepraemurium* Predominance of small lymphocytes HE × 380.

B Paracortical area of the popliteal lymph node of a C57/BL mouse 27 days after inoculation. Increased number of blastoid cells and groups of pale epithelioid cells. Methyl green pyronine × 380.

C Paracortical area of the popliteal lymph node of a C57/BL mouse showing groups of epithelioid cells. Mitosis in a blastoid cell (arrow) Methyl green pyronine 600

D Multinucleated giant macrophages infiltrating the cortex of the lymph node of a C3H mouse 65 days after inoculation. HE × 380.



mals, no bacilli were found in the popliteal lymph node after 65 days (Fig 3 A and B)

Fig 1 shows that, at some time between 110 and 230 days after inoculation of bacilli into the foot pads of C3H mice, their popliteal lymph nodes increased in size and eventually became very large. These enlarged lymph nodes were pale and of a glassy appearance. Histologically the early phase of the infection in C3H mice was characterized by the ocurrence of large multinucleated giant macrophages near the marginal sinuses of the lymph node (Fig 2 D) Approximately 3 months after inoculation such cells often formed aggregates which infiltrated the lymph node. At this stage, a marked proliferation of bacilli was evident and, at the end of the experiment, the lymph nodes were replaced by numerous macrophages containing large numbers of acid fast bacilli (Fig 3 C and D) Small lymphocytes were scarce in most animals. The late development of the lymph node lesions in C3H mice revealed groups of plasma cells between macrophages loaded with bacilli.

Immunofluorescence Studies

The distribution of immunoglobulin-containing cells (Ig-containing cells) in the popliteal lymph node was studied using a direct immunofluorescence technique. Two weeks after inoculation, any apparent differences between the lymph nodes from the two strains of mice were not observed Most of the Ig containing cells were confined to a localized area near the capsule. Owing to the simple structure of these small lymph nodes, the exact relation of this collection of cells to either the medullary or the cortical region was difficult to assess exclusively on the basis of its localization in the section.

Four weeks after inoculation, the distribution and amount of Ig-containing cells were still the same in lymph nodes from C3H mice (Fig 4 A) In the reactively enlarged lymph nodes from C57/BL mice Ig-containing cells were seen to be scattered throughout the lymph node but did not appear to be distinctly increased in numbers (Fig 4 B) During the following weeks, only small changes in these distribution patterns were seen. Some increase in the number of Ig-containing cells was observed in the peripheral region of the lymph nodes in C3H mice after 11 weeks (Fig 4 C and E) but since the variation from one animal to another was considerable and the number of animals examined was small, no definite conclusion could be drawn from these observations. In C57/BL mice, the number as well as the distribution pattern of Ig-containing cells remained nearly unchanged (Fig 4 D and F)

DISCUSSION

In mycobacterial infections, an effective defence seems to depend largely upon the presence of specifically sensitized lymphocytes which activate the macrophages locally in the lesions (*Dannenberg et al.* 1968, *Turk* 1969) During cell mediated immune reactions, marked proliferation of pyroninophilic blast cells occur in the paracortical area of the draining lymph node (*Oort & Turk* 1965, *Parrot & deSousa* 1966) probably giving rise to a new population of small lymphocytes (*Parrot* 1967)

The present study has revealed that the response in the local lymph node after inoculation of MLM differs markedly in C57/BL

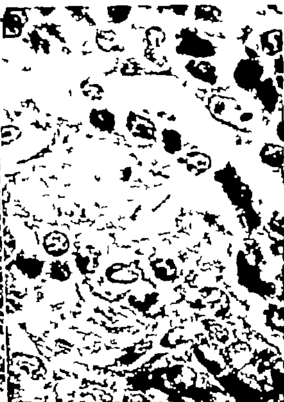
Fig 3 A Popliteal lymph node in a C57/BL mouse 230 days after inoculation showing groups of epithelioid cells, abundant small lymphocytes, occasional blast cells and some plasma cells. Methyl green pyronine × 380.

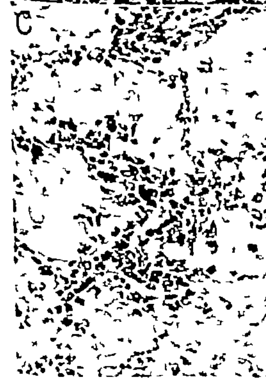
B Same lymph node as that shown in Fig 2 A Large epithelioid cell aggregate containing few and irregularly stained acid fast bacilli. Putt × 1500.

C Popliteal lymph node of a C3H mouse 230 days after inoculation, showing large numbers of macrophages which completely distort the lymph node architecture Large numbers of plasma cells are also seen between the macrophages. Methyl green pyronine × 380.

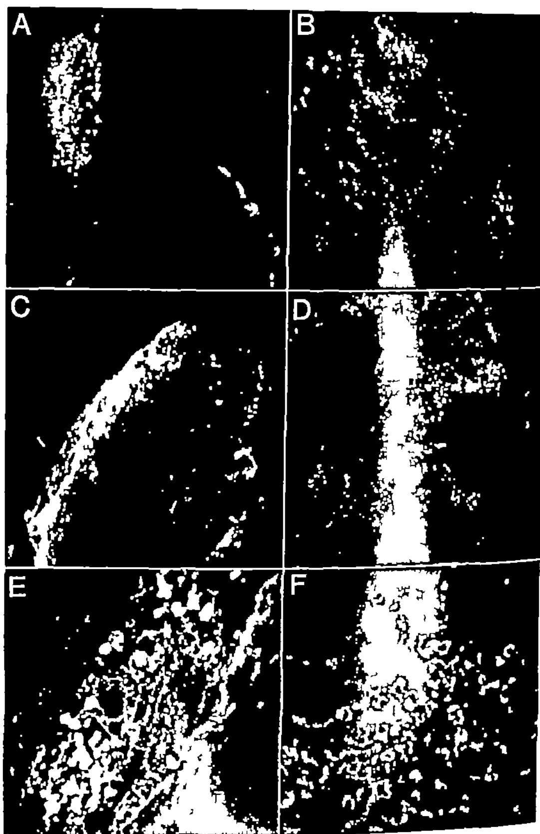
D Same lymph node as that shown in Fig. 2 C demonstrating distended macrophages heavily loaded with acid-fast bacilli. Putt × 1500.




B


C


D


A
B
C
D
E
F

nd C3H mice. Basically C57/BL mice show a adequate reaction including proliferation of blast cells in the T-cell area of the lymph node, development of epithelioid cell granulomas and no increase in the number of bacilli. These changes develop between two and four weeks after inoculation and coincide with the granulomatous reaction noted at the site of inoculation (*Closs & Haugen* 1975 a, *Haugen et al.* 1975, in preparation). In C3H mice, no reaction was seen in the draining lymph node until very late when several plasma cells were seen to surround infiltrates of macrophages loaded with bacilli. It may therefore be concluded that, while there is an early T-cell reaction in C57/BL mice the lack of any type of lymph node reaction in C3H mice is conspicuous. In particular there was no sign of an increase in numbers of Ig-containing cells in the early phase of the infection. Hence, the lack of T-cell response in this strain cannot be explained by an early B-cell reaction involving production of circulating antibodies which interfere with the development of cell mediated immunity.

It is well-known that lymph node enlargement often is observed in cases of long lasting leprosy (*Danielssen & Boeck* 1847) and that is more marked in lepromatous leprosy than in cases of borderline tuberculoid or polar tuberculoid leprosy (*Desikan & Job* 1966). The decreasing cell mediated immunity across the leprosy spectrum has been regarded as a secondary phenomenon, mainly due to the replacement of small lymphocytes by histiocytes in the paracortical areas (*Turk & Waters* 1969, 1971). Similarly the general depression of cell mediated immunity in murine leprosy (*Ptak et al.* 1970) is believed to be a consequence of the infection itself. Infiltration of the lymphoid tissue by epithelioid cells is a characteristic feature in patients with BT and TT leprosy in which bacilli are few (*Turk & Waters* 1971), giving the nodes a sarcoid-like appearance. *Turk* (1971) has suggested that the epithelioid appearance of histiocytes at this stage probably reflects a peculiar balance between the amount of antigen present and the degree of cell mediated immunity.

The findings in the present study bear some resemblance to the lymph node changes observed in human leprosy. The largest lymph nodes observed were those in C3H mice examined 230 days after inoculation when the lymph node was replaced by large macrophages heavily loaded with bacilli. At this stage the lymph nodes in C57/BL mice contained varying numbers of epithelioid cell infiltrates with few and mostly irregularly stained bacilli and abundant lymphocytes. With regard to lymph node pathology C57/BL mice evidently closely mimic the situation in the BT TT region of human leprosy while C3H mice resembles the advanced stage of human lepromatous leprosy. It may thus be concluded that the two strains C3H and C57/BL are polar also with regard to the lymph node reaction against MLM infection.

Fig. 4. Popliteal lymph nodes in mice injected with *M. lepraemurium* into the ipsilateral foot-pad. Stained with MRITC-labelled rabbit immunoglobulin against mouse immunoglobulins (Ig).

A. C3H mouse 4 weeks after inoculation. Note the follicle-like localization of Ig-containing cells. × 90.

B. C57/BL mouse 4 weeks after inoculation. Note the large size of the lymph node and the scattered appearance of Ig-containing cells. × 90.

C. C3H mouse 11 weeks after inoculation. The Ig-containing cells are somewhat increased in number but are still located to the periphery of the node. × 90.

D. C57/BL mouse 11 weeks after inoculation. The node is greatly enlarged, but the number of Ig-containing cells is not appreciably increased and show the same scattered distribution as that seen in B. × 90.

E. Same preparation as in C. Note that some of the fluorescence is not intracellularly localized. × 500.

F. Same preparation as in D. Not all of the fluorescence is distinctly intracellular. × 500.

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EXPERIMENTAL MURINE LEPROSY

8. *Ultrastructural Features of the Inflammatory Exudate and Bacterial Morphology in C3H and C57Bl Mice after Foot Pad Inoculation with* Mycobacterium lepraemurium

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Haugen, O. A., Skjorten, F. & Closs, O. Experimental murine leprosy. 8. Ultrastructural features of the inflammatory exudate and bacterial morphology in C3H and C57BL mice after foot-pad inoculation with *Mycobacterium lepraemurium*. Acta path. microbiol. scand. Sect. A, 83: 693–703, 1975.

Mice of the inbred strains C57BL and C3H were inoculated in the foot-pads with *Mycobacterium lepraemurium* (MLM) and the inflammatory reaction was studied using light and electron microscopy. In C57BL mice a granulomatous reaction developed 3–4 weeks after inoculation. The inflammatory exudate at this stage showed numerous lymphocytes, monocytes and macrophages. The latter cell type often contained many lysosomes and appeared activated. The bacilli which were all within phagosomes showed extensive electron dense aggregates of the cytoplasm suggesting severe damage. Lymphocytes and macrophages in close contact with eachother were often observed. In macrophages which contained damaged bacilli, spherical lipid-like bodies surrounded by granular endoplasmic reticulum were observed. It is suggested that this cell product could be of some significance for the bactericidal function of the macrophage. Contrary to these findings, the cellular infiltrate developing in C3H mice showed no lymphocytes and consisted exclusively of macrophages. These were all heavily loaded with bacilli. The vast majority of bacilli encountered in this strain was morphologically intact and presumably viable. Lipid-like bodies similar to those observed in infected C57BL macrophages were not encountered in C3H mice. It is concluded that unless the infected macrophages become immunologically activated they are unable to cause bacterial damage or to inhibit the growth of MLM.

Key words: Leprosy, murine, experimental; inflammatory exudate; ultrastructure; bacterial morphology.

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Mice vary considerably in their ability to resist infection with *Mycobacterium lepraemurium* (MLM) (*Kawaguchi* 1959, *Closs & Haugen* 1974). In C57BL mice the granulomatous reaction which develops at the site of inoculation prevents further dissemination of the infection. In C3H mice, the infection proceeds without any detectable host reaction (*Closs & Haugen* 1975 a, b). The granulomatous reaction coincides with the onset of systemic immunity (*Closs* 1975 b) and may

probably be regarded as a delayed type hypersensitivity reaction against bacterial antigens inducing activation of the macrophages at the inoculation site.

The extent and nature of the damage inflicted upon MLM in the resistant host is unknown. MLM is highly resistant to killing by phagocytes (*Rees et al.* 1960, *Allen et al.* 1965, *Brown & Draper* 1970). This might explain the chronic and progressive course of the infection in mice and rats (*Krakower & Gonzales* 1940) and indicates that MLM possesses properties not found in other mycobacteria which enable the bacterium to survive within the phagolysosomes of the macrophages (*Draper & Rees* 1970, *Hart et al.* 1972). The successful growth of MLM inside host cells *in vitro* and the apparent failure of phagocytic cells to effectively kill and degrade MLM *in vivo* (*Yang & Skinsnes* 1969) may however be due to lack of immunological activation of the macrophages. The main purpose of the present investigation was to study the ultrastructural features of the inflammatory exudate and bacterial morphology in C3H and C57BL mice infected with MLM at a stage when an immunological reaction had occurred in the highly resistant strain and the bacilli had ceased to multiply (*Closs* 1975 a).

MATERIALS AND METHODS

Experimental animals. Specific pathogen free (spf) female mice of the inbred strains C3H/Tif and C57BL/6J were purchased from Gl. Bomholdtgård Ltd, Denmark. The animals weighed 14–18 grams at the start of the experiments. They were kept in cages, 20 mice in each, from the start of the experiments; they were fed on pellets (Norwegian Standard Stock No. 1 Mice and Rats) and allowed tap water *ad libitum*.

Propagation of bacilli. M. lepraemurium, the Douglas strain (MLM) was propagated in the outbred strain NMRI and harvested as previously described (*Closs & Haugen* 1974).

Inoculation procedure. 10 microlitres of a bacillary suspension containing 2 × 10⁹ bacilli per ml were injected superficially into each of the hind foot pads. To prevent leakage of the inoculum, the injection site was sealed with plastic spray (Nobecutan, Bofors®, Sweden).

Histological examination. Four animals of each strain were killed 2, 8, 15, 27 and 41 days, respectively after inoculation. Both hind foot-pads together with the popliteal lymph nodes, inguinal lymph nodes, spleen and liver were removed. Using a small scissor the skin and subcutaneous tissue were dissected free from the metacarpal bones and adjacent plantar fascia. Tissues were fixed for conventional histology as previously described (*Closs & Haugen* 1975 a).

Electron microscopy. Small blocks were cut from the tissue removed from the foot-pads by means of razor blades. Handling of these specimens was performed in ice cold 2 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and fixation for 2 hours followed. Then the specimens were rinsed in the same buffer overnight and postfixed in 1 per cent osmium tetroxide in 0.1 M phosphate buffer, pH 7.4 for 1½ hours. Dehydration in graded ethanols was followed by embedding in Epon 812. Sections were cut with glass or diamond knives on LKB ultratomes. Semithin sections were stained with toluidine blue. Thin sections were stained with uranyl acetate and lead citrate, mounted on copper grids and examined in a Zeiss EM 9S electron microscope. Micrographs were taken at 1400–29000 × primary magnification and enlarged photographically as desired.

Criteria for Evaluation of Bacterial Morphology

Bacteria were classified into 3 morphological groups (*Armstrong & Hart* 1971, *Hart et al.* 1972).

1. *Intact bacilli* which contained a preserved nuclear region and did not show signs of cytoplasmic defects or condensations.
2. *Damaged bacilli* where the cytoplasmic structures were not preserved and cytoplasmic material was present as dense aggregates (*Rees & Valentine* 1962).
3. Bacilli which could not be classified as either 1 or 2 were classified as "*doubtful*".

Statistical methods. Comparison between proportions was performed by an χ^2-test (*Armitage* 1970).

RESULTS

Histological examination revealed that, during the first 8 days after inoculation, the inflammatory exudate consisted of numerous polymorphonuclear leucocytes (PML) and mononuclear cells with the appearance of monocytes. The infiltrates were similar in both strains of mice at this stage. Bacilli were found intracellularly both in PML and in monocytes.

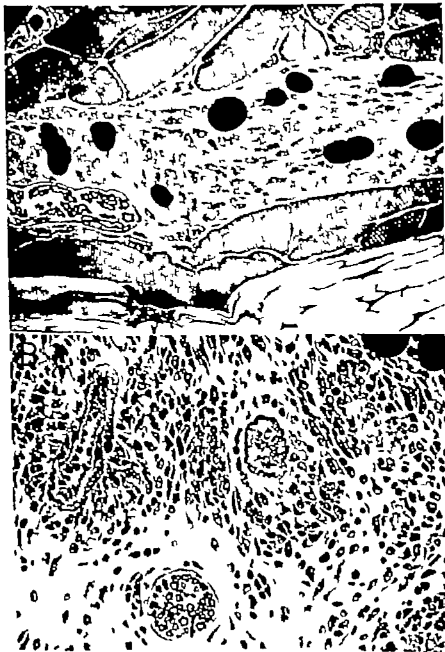


Fig. 1 *A.* Foot-pad infiltrate in C3H mouse 41 days after inoculation of MLM showing a large collection of macrophages between muscle bundles and fat cells. No other inflammatory cells are present. Toluidine blue stained Epon-section × 350

B. Foot-pad infiltrate in C57Bl mouse 41 days after inoculation of MLM showing a pleomorphic cellular infiltrate dominated by various types of mononuclear cells. Toluidine blue stained Epon-section × 350.

During the following week, the *infiltrate* decreased in size and consisted at this stage exclusively of large mononuclear cells containing many acid-fast bacilli. Other cell types were not encountered.

Twenty seven days after *inoculation* all C57BL mice showed extensive infiltrates consisting of lymphocytes and masses of mononuclear cells some of which resembled epithelioid cells. There was no tendency towards abscess formation or caseation. Bacilli were rather scarce and confined to the *cytoplasm* of some of the mononuclear phagocytes. In distinct contrast to these findings, the lesions of C3H mice showed no signs of inflammatory reaction. The lesion at this stage consisted of pale, large macrophages which all contained many acid fast bacilli and which produced a band shaped *infiltrate*. Other cell types were not encountered in the infiltrates. No plasma cells were found.

In both strains, histological examination of the tissues removed 41 days after inoculation revealed essentially the same findings as after 27 days (Fig. 1).

Electron Microscopic Findings

Blocks cut for electron microscopy had been collected as from the second day after inoculation. In semithin sections stained with toluidine blue bacilli and inflammatory cells were searched for but suitable specimens from both strains were not found until 41 days after inoculation.

Ultrastructural Features of the Infiltrate in C3H Mice

The uniform composition of this *infiltrate* as suggested by light microscopic examination was confirmed: the cellular infiltrate consisted of a homogeneous population of macrophages which all contained bacilli within their cytoplasm (Fig. 2 A). The bacillary load was approximately similar in all cells examined. Macrophages without bacilli were not encountered. Other cell types were not found.

The macrophages showed abundant microvilli, some mitochondria and scattered, membrane bound dense particles which presumably represented lysosomal structures. Bacilli were always within vacuolar sacs and surrounded by a clear space (Fig. 2 A-C). The capsular area of bacilli were more electron dense than the vesiculated or membraneous cytoplasm which was clearly seen in longitudinally sectioned microorganisms (Fig. 2 C). Some vacuoles contained more than one bacillus and some microorganisms looked as if they had been through a stage of division (Fig. 2 B). In connection with vacuoles containing bacilli fibrillar material was frequently observed, some of which could also be found in the cytoplasm, apparently without any relation to bacterial structures (Fig. 2 B and C). Occasionally elongated bacilli with marked swelling of the cytoplasm were found, the cytoplasms containing scattered small electron dense aggregates. These were counted as damaged and probably represented degenerate bacilli present in the original inoculum.

Ultrastructural Features of the Infiltrate in C57BL Mice

In C57BL mice the inflammatory exudate was composed of numerous macrophages and other mononuclear cells (Fig. 1 B). The majority of these cells did not contain bacilli, but in some of the cells, one, two or up to ten bacilli were found. In the central part of the infiltrate, however, occasional macrophages were observed which contained a larger number of bacilli. While some bacilli were morphologically intact, the morphology was obviously disturbed in the majority of them. Many appeared to be elongated and contained large rounded electron dense cytoplasmic aggregates

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Fig. 2. A. Numerous morphologically intact bacilli within phagocytic vacuoles of macrophages in a C3H mouse. × 9300.
B. M. lep*raemurium* within phagocytic vacuole in a C3H mouse. Note the less electron dense zone surrounding the bacilli and the fibrillar material (arrow) related to the phagosomes. × 24000.
C. Morphological intact MLM with vesicular structures of the nuclear membrane system. × 36000.

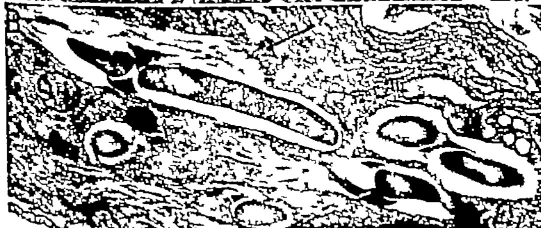






Fig 4 Lymphocyte in close contact with cytoplasmic projections of macrophage in a C57BL mouse Note "stimulated blebs" at the surface of the lymphocyte (arrow) × 12500.

peritoneal macrophages of immunized C3H mice has also been noted by *Yang & Skinsnes* (1969) However since bacterial multiplication and dissemination of the infection were not prevented, the morphological changes observed could not with certainty be ascribed to loss of viability

Intracellularly MLM and other mycobacteria are surrounded by an electron transparent zone (*Yamamoto et al.* 1958, *Chapman et al.* 1959) separating them from the unit membrane of the phagosome. The width of this zone is possibly increasing with time after infection (*Allen et al.* 1965) The material it contains is usually removed during dehydration and embedding, but it may also appear as a fibrillar material in the cytoplasm of the phagocytes (*Draper & Rees* 1973) This material which by now is identified as a mycoside of type C (*Draper & Rees* 1973) was presumed to have a protective function in separating the bacteria from the digestive enzymes of the host cell (*Draper & Rees* 1970 *Brown & Draper* 1970) According to *Hart et al* (1972) macrophages infected *in vitro* with MLM show fusion of lysosomes

and phagosomes whether the bacteria they contain are scored as intact or damaged. In the case of *M. microti* and *M. tuberculosis* however fusion with secondary lysosomes is mainly with damaged and less frequently with intact bacteria. Since lysosomal structures were not labelled in the present study we cannot make any comments upon this phenomenon. Bacilli as well as fibrillar material were less frequently observed in C57BL than in C3H mice and thus our findings neither prove nor disprove the concept that the fibrillar structures are related to a protective factor.

The tissue reaction and the ultrastructural features seen in C57BL mice after MLM infection have many similarities with the mouse foot-pad response to *M. leprae* (ML) (*Evans et al.* 1973) after inoculation of a suitable number of ML there is a lag phase upon which multiplication of bacilli takes place until a "ceiling" is reached. At this stage, when there is a granulomatous reaction at the local injection site, bacterial proliferation ceases and the organisms are killed (*Shepherd* 1960). *M. leprae* in activated macrophages is surrounded by a double membrane, the outer membrane showing unit membrane construction and representing the phagosome wall, while the inner is a single membrane in continuity with the bacterial wall. During the logarithmic phase however bacilli do not show phagosome membrane which apparently reappear at plateau (*Evans & Levy* 1972). In the present study we have not found any obvious differences as regards appearances of the phagocytic vacuoles or their construction in either strain or any relation of phagosome membrane to the morphological appearance of the phagocytized bacilli.

The proliferation of lymphoid blast cells in the paracortical area of the draining lymph node in C57BL mice (*Haugen & Closs* 1975) and the occurrence of lymphocytes at the site of inoculation 3–4 weeks after challenge with bacteria (*Closs & Haugen* 1975 a, b) are obviously of major importance for the outcome of the infection. The recruitment of mononuclear phagocytes into sites of delayed hypersensitivity reaction depends largely upon substances released from sensitized lymphocytes (*Jones* 1973, *Page et al.* 1974). Macrophage-activation is probably the most important factor in bacterial killing (*Dannenberg et al.* 1974) but the exact mechanism by which mycobacteria are killed is not clear. The infiltrate of C57BL mice showed several lymphocytes which appeared to be activated and in close contact with macrophages. In relation to such lymphocytes, structures resembling the "vesiculated blebs" described by *Biberfeld* (1971) in lymphocytes stimulated *in vitro* with PHA were frequently encountered. The significance of this observation is uncertain, but it may be related to the process of macrophage-activation. The significance of the spherical, lipid-like bodies observed in the cytoplasm of those C57BL macrophages which contained damaged bacteria also remains obscure. It is conceivable, however that they are synthesized by the cell since they were surrounded by granular endoplasmic reticulum. Furthermore, the fact that they were observed only in cells which contained damaged bacteria might indicate that they in some way are related to the process of microbial killing or degradation.

MLM has been regarded as a highly successful intracellular parasite which, without causing any damage to the host, can survive and multiply uninhibited within macrophages and other types of cells (*Chapman et al.* 1959, *Rees & Waters* 1962, *Allen et al.* 1965, *Brown & Draper* 1970). In C3H mice in which no immune reaction develops, the majority of bacteria phagocytized also remain morphologically intact. On the other hand, in C57BL mice, in which a granulomatous tissue reaction develops 3–4 weeks after a local injection of bacilli MLM is severely damaged. Thus, it appears that this microorganism will remain intact unless the macrophage becomes immunologically activated.

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DEOXYRIBONUCLEIC ACID CONTENT IN THE LARYNGEAL MUCOSA WITH SPECIAL REFERENCE TO POLYPLOID CELL NUCLEI

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Greisen, O Deoxyribonucleic acid content in the laryngeal mucosa with special reference to polyploid cell nuclei. Acta path. microbiol. scand. Sect. A, 83 704–708, 1975

The DNA content was measured by microphotometry in morphologically normal squamous epithelium of the larynx, in hyperplastic epithelium of the vocal cords and in invasive squamous-cell carcinoma. The average nuclear DNA content in the squamous-cell carcinoma was elevated in comparison with that of normal epithelium, and the values showed a much wider dispersion. During premitotic DNA synthesis, the nuclear content of DNA is doubled. Polyploid cell nuclei with a nuclear DNA content of more than twice the model value (the stem cell of the tumour) are therefore a distinct sign of an abnormally elevated content of DNA. In morphologically normal epithelium, no polyploid cell nuclei were revealed, while such nuclei were present in all the cases of invasive squamous-cell carcinoma. Polyploid cell nuclei were found in six out of 20 patients with hyperplasia of the epithelium of the vocal cords. Among these six patients, manifest invasive carcinoma developed in three, carcinoma in sit in one, while the condition remained benign in two, probably because the pathological tissue was completely removed The presence of polyploid cell nuclei should thus arouse a strong suspicion of malignancy and calls for close supervision of the patient.

Key words DNA laryngeal mucosa polploid cell nuclei,

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In normal tissue, the nuclear DNA content is distributed around a basal diploid value. In preparation for mitosis, DNA is synthetized until the DNA content reaches twice the normal diploid value. Variations in the nuclear DNA content is thus intimately connected with the mitotic activity

Cell nuclei in carcinoma are characterized by a considerable dispersion of the amount of DNA from a basal model value (the stem cell of the tumour) up to twice this value, and often with a considerable number of cells with a still higher DNA content (polyploid cells) The average content of DNA in malignant tumour-cell nuclei is elevated in comparison with that of normal cells.

Polyploid cell nuclei are defined as nuclei with a chromosome number larger than normal (*DeRobertis et al.* 1965) and the presence of these nuclei in a tissue indicates an abnormal condition.

The purpose of this investigation was to study the frequency and significance of polyploid cell nuclei in laryngeal epithelial tissue, i.e. in morphologically normal squamous epithelium, in hyperplasia and in invasive squamous-cell carcinoma



Fig 1 Polyploid cell nucleus in squamous-cell laryngeal carcinoma (RNase, gallocyanin-chromalum × 330)

MATERIAL AND METHODS

The material studied was laryngeal biopsy specimens obtained from the vocal cords of six patients with normal squamous epithelium, 20 patients with varying degree of epithelial hyperplasia (31 specimens) and 13 patients with invasive squamous-cell carcinoma (glottic carcinoma, stage 1) None of the patients had received radiotherapy It was not possible to obtain biopsy specimens from healthy patients with normal vocal cords Morphologically normal squamous epithelium was obtained from patients who had some kind of benign pathological condition of the larynx (polyps, nodules)

The biopsy specimens were fixed in 2 per cent formalin water embedded in paraffin, cut into sections 7 μm in thickness and stained with gallocyanin-chromalum (*Einarson* 1951 *Kiefer* 1970) Before staining, the sections were treated with ribonuclease (RNase) for 2 hours at 37 C in a phosphate buffer pH 7 6 by this treatment the basophilia brought about by RNA disappears completely

The microphotometric measurements were made by a Leitz microphotometer with a measuring spot of 2.5 μm in diameter The light source was monochromatic light. Gallocyanin-chromalum in solution and gallocyanin-chromalum-stained nuclei have an absorption maximum at 570 nm. The nuclear extinction is often very high, and in order to obtain absorption values in an area where the photometer error is comparatively small, measurements of the nuclear extinction were made at some distance from the absorption maximum 510 nm was found to be appropriate (*G elsen* 1971) The DNA content was calculated according to the "plug method" as the product of area and extinction. A number of 30–50 cell nuclei were measured in each section to determine the basal modal value, and the number of polyploid cell nuclei was then counted per 1000–1500 cells.

With due allowance for premitotic DNA synthesis, polyploid nuclei are in microphotometry defined as nuclei containing DNA amount higher than twice the basal modal value on account of dispersion and measuring errors the limit is fixed at 2.5 times the modal value (Fig. 1)

The method used was the same as that applied in study of bronchogenic carcinoma (*G elsen* 1969 1971)

RESULTS

Squamous-Cell Carcinoma

The DNA content was measured in the cell nuclei of biopsy specimens from 13 patients with invasive squamous-cell carcinoma. A typical distribution pattern of the nuclear DNA content is shown in Fig 2.

From the basal model value the nu

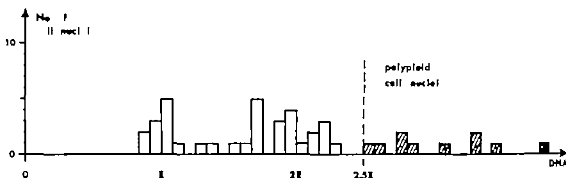


Fig 2 Distribution of DNA content and polyploid cell nuclei in squamous-cell laryngeal carcinoma.

tent is dispersed up to twice this value and even higher Polyploid cell nuclei were present in all squamous-cell carcinomas. In some cases, the nuclei exhibited very pronounced DNA synthesis with a DNA content of up to 12 times the basal modal value. The number of polyploid cells per 1000 nuclei and their maximum DNA content appear from Table 1

The average number of polyploid cell nuclei in squamous-cell carcinoma is thus 6.3 per 1000 cells, which is of the same order as in squamous-cell bronchogenic carcinoma (6 7 per 1000 cell nuclei *Greisen* 1971)

Hyperplasia

In 31 sections from 20 patients with a varying degree of hyperplastic epithelium of the vocal cords, polyploid cell nuclei were found in six patients, while no such nuclei were present in 14 (Table 2)

In the six patients *with* polyploid cell nuclei atypical nuclear changes of varying degree was revealed by ordinary microscopic examination. The patients were checked at regular follow up examinations up to 8 years. Invasive carcinoma subsequently developed in three of the patients with polyploid nuclei. In one, the dysplasia was severe on the verge of carcinoma in situ, but no signs of manifest carcinoma have appeared. In the two other patients, the hyperplastic mucosa of the vocal cord was completely removed by the endolaryngeal intervention. The vocal cords are still thickened in one of the latter two patients, but perfectly normal in the other

TABLE 1 *Polyploid Cell Nuclei in Laryngeal Carcinoma and Their Maximum Content of DNA in Relation to th Value of the Stem Cell (Modal Value)*

Section No.	No. of polyploid nuclei per 1000 cells	DNA content/ Modal value
8428	10 7	5 1
1375	2.7	3 9
7194	1.3	3.5
448	7	3.2
1969	5 7	12.1
11647	9 4	4 4
295	ـ7	3.3
3690	5 4	12.2
4898	20 8	7.2
478	3 4	4.2
585	8.7	7.2
4553	3.0	6.4
2006	5.0	9 3
Average	6.3	

Among the 14 patients *without* polyploid nuclei, invasive carcinoma developed in one but because of technical difficulties in the direct laryngoscopy the biopsy specimen obtained was undoubtedly non-representative in this case. In the remaining 13 patients, no signs of malignancy have appeared.

Normal Squamous Epithelium

In six patients with normal squamous epithelium, polyploid cell nuclei were not revealed in any of the specimens.

The occurrence of polyploid nuclei in various laryngeal tissues appears from Table 3.

TABLE 2. *Polyploid Cell Nuclei in Hyperplastic Epithelium*

	Development of carcinoma	Carcinoma in situ	No malignancy	Total
Polyploid cell nuclei	3	1	2	6
No polyploid cell nuclei	1	–	13	14

TABLE 3. *Polyploid Cell Nuclei in the Larynx of Patients with Normal Squamous Epithelium, Hyperplastic Epithelium and Invasive Squamous-Cell Carcinoma*

	No. of patients	Frequency of polyploid nuclei	
		No.	Per cent
Normal squamous epithelium	6	0	0
Hyperplastic epithelium	20	6	30
Squamous-cell carcinoma	13	13	100

DISCUSSION

Abnormal chromosomal content is a basic change in malignant tumours (*Koller* 1972). It is a characteristic feature that the average DNA content in the nuclei is high, and that the values show a wide dispersion from a basal modal value up to twice that level. Furthermore there is often an appreciable number of cell nuclei with even higher values (polyploid cell nuclei).

In the range from the basal modal value up to twice that level, it is not possible by microphotometry to distinguish cells with an aneuploid content of chromosomes and elevated DNA content from cells synthetizing DNA in preparation for mitosis. In tumour tissue, however, DNA values above twice the basal modal value do have a definitely elevated DNA content. In comparison, it may be mentioned that these polyploid cells characteristically were found in bronchogenic carcinoma, and only in a few cases of metaplastic bronchial epithelium with nuclear polymorphism (*Greuen* 1971).

Pleomorphic adenomas of the parotid gland of long duration pre-operatively are characterized by a small fraction of tetraploid cells, possibly of importance in the development of malignancy, as it has been found that carcinoma in pleomorphic adenomas also have tetraploid fraction (*Eneroth* & *Zetterberg* 1974).

A histological assessment and grading of the malignancy of carcinoma of the larynx based on *morphological* criteria were used by *Jacobsson et al.* (1973). They included nuclear polymorphism and the number of enlarged cell nuclei in their assessment.

In the present *microphotometric* investigation of laryngeal epithelial tissue, polyploid cell nuclei were never found in morphologically normal squamous epithelium, but invariably in invasive squamous-cell carcinoma. In the intermediate group of 20 patients with epithelial hyperplasia of the vocal cords, polyploid cell nuclei were found in six patients, including three in whom malignancy eventually developed.

The presence of polyploid cell nuclei in the laryngeal epithelium is thus in itself a sign which must give rise to suspicion of subsequent development of malignancy and therefore calls for close supervision of the patient. On the other hand, the absence of polyploid nuclei in a biopsy specimen does not rule out the possibility of subsequent malignant development as the specimen studied may not have been representative.

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GROWTH PATTERNS AND CHROMOSOME CONSTITUTIONS OF HUMAN MALIGNANT TUMOURS AFTER LONG TERM SERIAL TRANSPLANTATION IN NUDE MICE

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Povlsen, C. O. Visfeldt, J. Rygaard, J. & Jensen, Grethe. Growth patterns and chromosome constitutions of human malignant tumours after long-term serial transplantation in nude mice. Acta path. microbiol. scand. Sect. A, 83 709–716 1975

Fi human malignant tumours, heterotransplanted to athymic nude mice, have been re-investigated after long-term serial transplantation, and their growth patterns and chromosomal constitutions compared with their early appearance. After 27–56 passages over 3/ –3½ years, all of the tumours—two adenocarcinomas of the colon, two malignant melanomas, and one Burkitt' lymphoma—retained the cytological and histological ppearance of the inoculated human material. All the mitoses observed in the chromosome studies were of human karyotype. The fact that no total chromosomal species shift, no interspecies hybridization, and no changes in biological properties ppeared—as observed in some other heterotransplantation systems—would indicate that human tumours grafted to nude mice are more suitable models in, e.g. studies concerning cancer chemotherapeutics.

Key words: Heterotransplanted human tumours nude mice growth pattern chromosome analyses.

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Since 1969 a number of human malignant tumours have been transplanted successfully to the mouse mutant nude which suffers from thymic aplasia (*Pantelouri* 1968) It has been possible to carry out serial transplantation through many passages in new nude recipients (*Rygaard & Povlsen* 1969 *Povlsen & Rygaard* 1971 1972, *Povlsen et al* 1973 *Giovanella et al.* 1974 *Sordat et al* 1974 *Povlsen* 1976)

Will such heterotransplanted tumours, following long term serial growth in nude mice, maintain or change their original biological properties?

Changes have been demonstrated in human tumours transplanted to the cheek pouch in the Syrian hamster (*Lampert et al* 1968, *Goldenberg et al.* 1971 *Goldenberg et al* 1974) in cortisone-treated mice (*Iversen* 196?) and intraembryonally in mice and rats (*Popescu et al* 1970) The changes manifest

themselves in a more aggressive growth of the tumours in the host, the tumours gaining capacity for metastatic spreading to lymph nodes and organs. Chromosome analyses of these tumours have occasionally revealed total chromosomal species shift (*Iversen* 1962, *Lampert et al.* 1968, *Popescu et al.* 1970) and in other cases signs of inter-species hybridization were observed (*Goldenberg et al.* 1974).

In previous reports we have described growth patterns and results of chromosome studies of early passages of a number of human malignant tumours transplanted to the mouse mutant nude (*Povlsen & Rygaard* 1971, 1972, *Visfeldt et al.* 1972, *Povlsen et al.* 1973).

The object of the present study was to analyze the morphology and growth pattern of five tumours, which have previously been studied, as well as the chromosomal pattern following many transplantations in the mouse mutant nude. On the basis of these studies, and a review of the literature, the importance of species shift or inter-species hybridization for the biological properties of the tumours and the applicability of the model is discussed.

MATERIAL AND METHODS

Mice. The experiments were made on 6 to 8 weeks old nude mice of both sexes, bred at the Institute of Pathology, Kommunehospitalet, Copenhagen. These mice were the direct progeny of 4–9 back-cross cycle in a gene transfer to BALB/c mice, (*Rygaard* 1973).

Tumours. The investigations were performed on five different malignant human tumours which have been serially transplanted in nude mice through 27–36 passages so far (see Table 1) over periods varying from 3½ to 5½ years. The origin of the tumours, their histological appearance and chromosomal pattern during early passages in nude mice have previously been described (*Visfeldt et al.* 1972, Nos. 1–3, 5–6, 7 and 12, 13, *Povlsen et al.* 1973) and these features are included in Table 1 for the purpose of comparison with subsequent chromosome studies. The methods of inoculation used are described in a previous paper (*Povlsen & Rygaard* 1971).

Observation of the tumour growth. After inoculation, the growth of the tumour was assessed every day and the animals were examined with a view to revealing metastases of the tumours. In all animals autopsy was performed, including examination of regional lymph nodes as well as thoracic and abdominal organs for metastases. For each of the 5 types of tumour 1–3 tumours were selected at random from each individual transplantation generation for histological examination. Furthermore, tissue was removed for microscopical study from enlarged regional lymph nodes and organs with gross changes. The tissue specimens were fixed in formalin, embedded in paraffin and sections of 7 μm were stained with haematoxylin and eosin.

Chromosome studies. Tumours, 6 to 10 mm large, were removed for chromosome analyses, usually 6 weeks after inoculation. The analyses were carried out by a direct method, as previously described (*Visfeldt et al.* 1972). The number of serial transplantations in the nude mouse at the time of analysis is shown in Table 1.

RESULTS

The Macroscopic and Microscopic Growth Pattern of Transplanted Tumours in the Mouse Mutant Nude

The rate of growth of the various tumours in the nude mouse varied greatly but, in each individual tumour the growth rate was rather constant and did not change from one serial transplantation to another. In all cases the growth of the tumour in the nude mouse was localized and gross or histologic metastases to lymph nodes or organs were not observed. At microscopical examination 3 of the tumours (2 colon carcinomas and 1 malignant melanoma) were well-defined and surrounded by a dense accumulation of connective tissue resembling a capsule (Fig. 1), whereas 2 of the tumours (1 malignant melanoma and 1 Burkitt's lymphoma) were locally invasive in fatty tissue and striated muscle (Fig. 2). This pattern of growth was constant throughout the serial transplantations. In all cases the tumours developing in the nude mice maintained a cytological and histological appearance which was in full accordance with that of the inoculated human material (Figs. 3 + 4).

Chromosome studies. The results of the chromosome studies are presented in Table 1. Thorough chromosome analysis was carried out in 2 of the tumours of this series (analyses

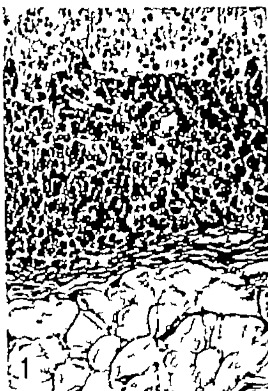
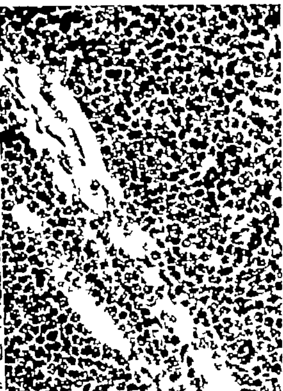



Fig 1 Section from marginal zone of human malignant melanoma (record no. P2736) serially grown in nude mice. The tumour is well-defined, separated from striated muscle (bottom) by a layer of connective tissue. 180 × H&E.

Fig. 2 Invasion of striated muscle by Burkitt's lymphoma (record no. I P 14858) transplanted to nude mouse. 350 × H&E.

Nos. 876 and 878) whereas, in the remaining cases, the assessment was based on screening. All the mitoses observed in the present material were of human karyotype (Figs 5 + 6).

The number of chromosomes in the cells studied varied greatly from one tumour to another. For each individual tumour there were some variations, however the chromosome number falling within the same range in early and late transplantation generations.

DISCUSSION

Growth Pattern of the Tumours

The results found during the present study show that human malignant tumours, after transplantation to nude mice, present a macroscopically and microscopically constant pattern of growth throughout up till now 27–56 serial transplantations in nude mice over periods varying from 3½ to 5½ years. The tumours present local growth at the site of implantation without any tendency to metastatic spread to lymph nodes or organs.

These results are in accordance with studies carried out by *Sordat et al.* (1974) concerning a number of human tumours inoculated directly into nude mice of BALB/c background. Conversely *Giovanella & Stehlin* (1974) injecting cell cultures derived from human malignant tumours into outbred C57 BL/6 and Swiss nude mice found development of tumours in these animals with local invasion and metastases to lymph nodes. Chromosome analyses of these tumours revealed human karyotype. Likewise, by direct inoculation of 4 human malignant tumours nude mice *Giovanella et al* (1974)






Fig. 5 Metaphase from Burkitt lymphoma (record no. I.P 14858) after 18 passages in nude mice. Human karyotype with 46 chromosomes.

Fig. 6 Metaphase from poorly differentiated adenocarcinoma of the colon (record no. 20961) after 45 passages in nude mice. Human karyotype with 46 chromosomes.

invasively growing tumours, in one of the cases with metastasis to a regional lymph node. Chromosome analyses of these directly inoculated tumours were not performed. The reasons for this discrepancy in the growth pattern remain to be clarified. Possible explanations include differences in the genetic origin of the animals and their conditions of living (*Giovanella et al.* 1974 *Povlsen* 1976)

Fig. 3 a. Section from operative specimen (record no. 20746) showing mucoid producing adenocarcinoma.

Fig. 3 b Microscopic appearance of the tumour after 45 serial transplantations in nude mice. Note close similarity with Fig. 3 a. 140 × H&E.

Fig. 4 a. Section from operative specimen (record no. I.P 14858) showing Burkitt lymphoma with starry sky appearance.

Fig. 4 b Microscopic appearance of the tumour after 16 passages in nude mice. The histologic and cytologic picture is in accordance with Fig. 4 a. 350 × H&E.

Chromosome Type of the Tumour Cells

All the cells analyzed or screened presented human karyotypes. Hence the present material did not reveal hybridization of human and murine cells or total species shift.

Chromosome Number of the Tumour Cells

The variation in the chromosome number of the cells of the various tumours and the dominance of hyperdiploid cells is in full accordance with previous results (*Laufeldt et al.* 1972) There was good agreement between the chromosome number of the individual tumours in early and late transplantation generations.

TABLE 1 *Histological Appearance and Chromosome Stud*

Patient's record No.	Tumour material	Transfer No.	Chromosome analysis No.	1970–1971 Number of cells analyzed	Chromosome number of cells analyzed	Number of cells screened	Chromosome number of cells screened
20246	Well differentiated adeno-	6	450	0	–	4	–
–	carcinoma of sigmoid colon.	7	455	0	–	0	
–	Abundant production of mucin.	8	519	4	51(3) 52(1)	30	52 54
20961	Poorly differentiated adeno-	1	365	0	–	5	
–	carcinoma of the transverse colon.	2	389	1	45	5	–
P2736	Subcutaneous metastasis of left femur from malignant melanoma of left ankle.	1	452	1	52	9	54–77
P2800	Subcutaneous metastasis	1	542	0	–	2	68
–	from right femur from malignant melanoma of right lower leg	3	587	3	62(2) 66(1)	8	43–65
I.P 14858	Burkitt's lymphoma of lower abdomen including both ovaries.	3	–	40	46	200	46

* serial transplantation discontinued after transfer no. 37

Other Heterotransplantation Systems

By chromosome analysis of a human tumour transplanted to the cheek pouch of Syrian hamsters, *Goldenberg et al* (1974) found grounds for the assertion that, in this system an *in vivo* hybridization of human and hamster cells occurred. Indications of hybridization were found in 11 out of 13 serially grown tumours (*Mihich et al* 1974). This hybridization theory is supported by antigen analyses and isoenzyme studies (*Götz & Goldenberg* 1967 *Goldenberg et al* 1971).

In 1962 *Ivarsen* carried out chromosome analyses of human tumours transplanted to cortisone treated mice. In one case tumours with human karyotype were found in the first transplantation generation whereas, in the 15th transplantation generation chromosomes of murine type only were found. Likewise *Popescu et al* (1970) revealed total species shift when studying a tumour of human origin transplanted intraembryonally in rats and mice.

On the basis of the above studies and our own results obtained in the mouse mutant nude, it seems justifiable to presume that hybridization of the tumour cells could be the cause of the changed biological properties of the cells, making the development of metastases possible.

The fact that no total chromosomal species shift, no inter species hybridization, and no changes in biological properties appeared—as observed in some other heterotransplantation systems—would indicate that human tumours grafted to nude mice are more suitable models in, e.g., studies concerning cancer chemotherapeutics.

		1974				
Transfer No.	Chromosome analysis No.	Number of cells analyzed	Chromosome number of cells analyzed	Number of cells screened	Chromosome number of cells screened	Transfer No (apr 1975)
33	877	0	–	6	50-60	45
45	876	6	36 (1) 45 (2) 46 (3)	6	46	56
37	889	0	–	50	48-76	
28	879	0	–	13	60 65	40
18	878	10	44 (2) 45 (2) 46 (6)	40	42-47	27

** 43 cells with chromosome number 70 76

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THE EFFECT ON THE RAT LIVER OF LONG TERM ADMINISTRATION OF DIFFERENT ALCOHOLIC BEVERAGES TOGETHER WITH INADEQUATE DIETS

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Jordö, L. & Olsson, R. The effect on the rat liver of long-term administration of different alcoholic beverages together with inadequate diets. Acta path. microbiol. scand. Sect. A, 83 717-720, 1975

Rats were given 36 per cent of calories as ethanol, gin, brandy whisky or red wine together with hypocaloric (25 per cent of normal) hypocaloric—low-protein—highfat, or hypocaloric—low vitamin diets for several months and compared with rats given isocaloric amounts of glucose instead of alcohol. In spite of high mortality rate no severe liver lesions occurred, especially no cirrhosis. Congeners present in different alcoholic beverages therefore seem to lack important hepatotoxic effects at least in the rat.

Key words Liver rat alcohol inadequate diets

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Nutritional imbalances have been considered as causative factors for the development of liver cirrhosis in the human alcoholic. Animal experiments designed to elucidate the importance of nutritional imbalances have usually resulted in equally pronounced hepatic changes in alcohol animals and control animals given sugar instead of ethanol (8). The conclusions to be drawn from such experiments are thus that ethanol is as hepatotoxic as sugar and that the cirrhotic changes are wholly attributable to nutritional imbalances. However severe nutritional deficiencies in man do not lead to cirrhosis (2, 10 11 12). It therefore seems more probable that the alcoholic beverages play an important role in the production of liver cirrhosis in the alcoholic.

We have investigated the possibility that the cirrhogenic effect is attributable to other substances than ethanol present in alcoholic beverages (4). However although biochemical analyses did present some support for the hypothesis that different alcoholic beverages may vary in their liver-damaging effect, histological studies failed to reveal more than minor alterations in the liver parenchyma.

In view of the fact that human alcoholics often have a very poor food intake it was considered of interest to extend the above mentioned investigations to a histological study in which the chronic administration of different alcoholic beverages was combined with grave nutritional deficiencies.

METHODS

Male Sprague Dawley rats, weighing 150 g at the start of the experiments were used. They were housed in bottom wired cages and given liquid diets as the only source of food and water.

The main purpose of this investigation was initially to study the effect of different alcoholic beverages in rats given a hypo-caloric, but otherwise well-balanced diet. In order to enable an exact presentation of administered nutrients a synthetic diet of known composition was used. With some modifications, the diet described by *Lieber et al.* (5) (Table 1) was used. The salt formula of *Hegsted et al.* (3) was used, but the vitamin mixture was that described by *Porta et al.* (8) with the addition of choline in the same concentration as used by *Lieber et al.* (5). The "lipotrophic value" of the diet (7) was 125 mg choline/100 cal. The amino acid mixture (AB Astra, Södertälje, Sweden) contained (g/100 g mixture) L-arginine chloride 7.89 L-phenyl alanine 6.42 L-histidine monochloride 4.37 L-isoleucine 4.09 L-leucine 6.42, L-lysine monochloride 5.82, L-methionine 6.42, L-threonine 2.92 L-tryptophan 1.47 L-valine 4.66 amino acetic acid 49.6. Fat was incorporated as an oil mixture containing by weight, 5 parts of corn oil 1.5 parts of cod liver oil and 12 parts of olive oil. Manucol SS 70 was used to stabilise the liquid diet.

Alcohol was isocalorically given as ethanol gin, whisky brandy and red wine of cheap qualities. In a control group alcohol was isocalorically replaced with glucose. The daily consumption was 12–15 calories/rat/day corresponding to about 4 g alcohol/kg/day.

Each group initially contained 20 rats. After 12 weeks a liver biopsy was performed under ether anaesthesia in three animals from each group (series A). Because of the very slight histological changes, the protein content of the diet was reduced to 16 g/L, (10.9 per cent of total calories) increasing the percentage caloric intake from alcohol to 39.6 from fat to 43.7 and from glucose to 5.8. The "lipotrophic value" of the diet was 75 mg choline/100 cal. The surviving animals were sacrificed after a further 10 weeks (series B).

Since also these animals showed very slight histological changes, another series of animals (C) were given the same diet as series A except for the vitamin mixture which was reduced to 1.0 g/L. Each group initially contained 17 rats except for the brandy group that contained 16 rats. After seven weeks, a biopsy through laparotomy was performed in two rats from the brandy group and in three rats from each of the other groups. All these animals died at or shortly after the operation. After 11 weeks a new biopsy was performed in all the surviving animals. Two ethanol rats and one whisky rat died at or following the laparotomy.

TABLE 1 *Composition of the Semiliquid Diet Given to the Rats in Series A*

	g/L	Calories (% of total)
Sucrose	8.4	5
Fat	30.8	39
Amino acids	31.5	20
Ethanol (96 %)	33.4	36
Vitamin mixture	2.0	
Salt mixture	5.0	
Choline chloride	0.167	
Manucol	1.0	
Water	922.5	

The diet contained 655 cal/L. The "lipotrophic value" (choline plus methionin) of the diet = 125 mg/100 cal. (calculated according to Porta & Gomez-Dumm (7))

All surviving animals were sacrificed after 15 weeks.

The samples of liver tissue taken at biopsy or at autopsy were fixed in 10 per cent neutral formalin and stained with van Gieson's stain.

The total number of animals in which a histological study of the liver was possible is given in Table 2. Because of autolysis or cannibalism, the livers from most of the spontaneously dead rats were not accessible to histological study.

RESULTS

In series A, the mortality was about 25 per cent in all groups. No weight gain occurred and many animals were in a poor condition. Histological examination revealed a slight to moderate fatty infiltration in some of the sections, mainly around the central veins. No clear differences were detectable between the groups. There was no inflammation, no necrosis and no increase of the fibrous tissue.

In series B the number of surviving animals were: controls 8, ethanol 9, red wine 4, brandy 2, gin 2, whisky 0. Some recently deceased rats were included in the histological study. The number of livers examined histologically is given in Table 2. A rather pronounced steatosis was seen in one gin rat, but otherwise there was no steatosis, necrosis, inflammation or increased fibrous tissue in any of the animals.

In series C no steatosis, necrosis, inflamma-

TABLE 2. *The Total Number of Animals in Which a Histological Study of the Liver Was Possible In Series C no Brandy or Gin Rats Survived 11 Weeks*

	Series A	Series B	Series C			
	Biopsy 12 w	Sacrifice 72 w	Dead 4–7 w	Biopsy 7 w	Biopsy 11 w	Sacrifice 15 w
Controls	3	8		3	8	3
Ethanol	3	6		3	6	
Brandy	3	5	7			
Whisky	3	4		3	4	3
Gin	3	5	2	3		
Red wine	3	6	1	3	4	4

tion or increase of fibrous tissue was visible in sections from animals that died after 4–8 weeks or in the biopsies taken after 7 weeks. In the biopsies taken after 11 weeks as well as at sacrifice after 15 weeks a slight to moderate fatty infiltration was sometimes seen, without clear differences between the groups. There were no necroses, no inflammation and no increase of fibrous tissue in any of the sections with the exception of one ethanol rat that displayed a few discrete necroses with lymphocytic infiltration.

DISCUSSION

Although the diets contained all amino acids essential for the rat (8) it was not tested whether the relative amount of the different amino acids were optimal for promoting growth in the rat. However the main cause of the lack of weight gain and high mortality in the animals of the present series probably the very low amount ... which was only about 25 per cent of the amount spontaneously ingested by ... of similar weight.

The lack of remarkable ... changes in the liver in the control ... in accordance with observation in ... humans with grave nutritional imbalance ... 10, 11, 12). Replacement of 1/3 of the daily caloric intake with alcohol did not provoke any further histological changes ... the liver. Many but not all hepatotoxic drugs are rendered more toxic when given in combination with deficient diets (1). Although the ethanol intake in the present experiment (4 g/kg/day) may not be great enough to permit definite conclusions, the present results do not suggest that ethanol and other compounds present in alcoholic beverages (so-called congeners) belong to this group of agents, for the diets used in the present study were hypocaloric, low protein, highfat and/or low-vitamin diets (8).

The possibility that the deficient diets have instead decreased possible hepatotoxic effects of the congeners cannot be completely excluded. It is known that severe protein deficiency decreases the hepatotoxic effect of those drugs (e.g. CCl_4) which are metabolized in the microsomes before rendered toxic (6). However it is noticeable that only very slight changes were induced in rats given 50 per cent of calories as hard liquors for 8–9 months but an otherwise well-balanced diet (4).

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COLLAGEN DEVELOPMENT IN GRANULATION TISSUE AS COMPARED WITH COLLAGEN OF SKIN AND AORTA FROM INJURED AND NON INJURED RATS

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Granulation tissue in rats was produced by subcutaneous implantation of viscose cellulose sponges. Granulomas, aortae, and skin samples were taken 4 8, 14 22, 33 and 42 days after the sponge implantation and compared with age-matched non-operated rats. ^{14}C-proline was given 4 hours before death to animals killed on day 0 14 and 42. The ^{14}C-OH-proline activity in salt insoluble collagen was higher in granulation tissue and aorta than in skin. This indicates a faster formation, or an increased stability of the intermolecular cross-links in granulation tissue and aorta, than in skin. The percentage of free OH-proline was higher in granulation tissue than in skin, reflecting a relatively increased collagen degradation in granulation tissue. An increased collagen degradation may also, in part, explain a registered higher alpha/beta ratio in collagen from granulation tissue than from skin, as well as the increase in alpha/beta ratio in the older granulomas. The sponge implantation did not affect the collagen of aorta and skin, but caused a decrease in the dry weight of aorta and skin, and an increase in the number of granulocytes in the blood.

Key words Collagen granulation tissue skin aorta.

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Recent years have brought evidence of biochemical differences between collagens from various tissues These differences include variations in the amino acid composition, the degree of hydroxylation of proline and lysine, the degree of glycosylation of OH lysine, as well as differences in the type and stability of the intermolecular cross-links in collagen (Nimni 1973 *Bailey et al.* 1974 *Bornstein* 1974) The knowledge of these tissue differences may lead to a better understanding of differences in the reactivity of various tissues, and may also open the possibility of a more specific pharmacological influence on the various connective tissues.

The purpose of the present investigation was to compare some aspects of collagen metabolism in aorta, skin, and granulation tissue of rats. It was furthermore intended to analyze if the local production of granulation tissue influences the collagen from the apparently intact aorta and skin of the same animal.

MATERIALS AND METHODS

One hundred and eight male Sprague-Dawley rats, 40 days of age, and weighing 140–150 g were used. Viscose cellulose sponges (Visella®) were implanted subcutaneously to produce granulation tissue. The sponges were washed thoroughly with water cut into pieces of 1 × 1 × 2 cm, and sterilized by boiling in physiological saline for 30 minutes before the implantation. The dry weights of the sponges were 80–90 mg

At the beginning of the experiment 48 rats were anesthetized with ether and each had 2 sponges implanted symmetrically under the superficial fascia in the infrascapular region, as described by *Viljanto* (1964) On the same day 12 non-operated animals were killed by decapitation. On the 4th, 8th, 14th, 22nd, 33rd, and 42nd day after the operation, 6–12 operated and 6–12 non-operated animals were killed by decapitation. The rats who were killed at the beginning of the experiment and on the 14th and 42nd day received 4 hours before they were killed 25 µCi of uniformly labelled L-^{14}C-proline (New England Nuclear) intraperitoneally in 1 ml of physiological saline containing 100 µg of non-labelled L-proline. Four hours of labelling *in vivo* was chosen, because the maximal activity of ^{14}C-OH-proline in salt soluble collagen is achieved during the first hours after ^{14}C-proline administration (*Jackson & Bentley* 1960 *Laitinen* 1967) At the decapitation blood was collected for determinations of hemoglobin concentration and leucocyte count, including a differential count of granulocytes and mononuclear cells.

The entire aorta from the aortic valve to the bifurcation was removed and the adventitia stripped off Two circular skin samples of 2 1 cm in diameter were taken symmetrically from the lumbar regions, 1–2 cm apart from the skin wound in the sponge-bearing rats. The hair was cut with an electrical hair cutter and the subcutaneous fat was removed with a scalpel. The granulomas were removed and freed from adherent connective tissue. The aorta, the 2 skin samples, and 1 granuloma were lyophilized at 4 C to constant weight, and the water content was determined as the difference between the wet and dry weight. The dried tissue was ground in a Wiley mill at 4 C. The total content of OH proline was determined in all 3 tissues after hydrolysis in 6 M HCl at 136 C for 6 hours. The OH-proline determinations were carried out according to the method of *Prockop & Udenfriend* (1960) as modified by *Juva & Ziff* (1962) Hydroxyproline is synthesized in vivo by hydroxylation of peptide-bound proline and is in tissues from mammals only found in collagen (13–14 g per cent) and in elastin (1–2 g per cent) (*Jackson & Cleary* 1967)

Collagen Fractionation

The collagen extraction procedures are summarized in the flow sheet in Fig. 1 The salt soluble collagen was extracted from samples of aorta, skin, and granulation tissue by 4 extractions overnight at 4 C with 5 ml of 0.5 M NaCl in 0.02 M Tris-HCl, pH 7 4 with one drop of octanol added as preservative. Between each extraction the soluble collagen was separated by centrifugation at 4 C for 30 minutes at 45,000 × g and filtered through Whatman filter grade 90. The OH proline content in the combined supernatants was taken as an index of salt soluble collagen. The OH-proline in the residue of skin and granulation tissue was taken to represent insoluble collagen, as 95–99 per cent of the OH-proline was found to be gelatinized after autoclavings with water at 128 C. In the aortic samples the insoluble collagen was recovered as gelatine by 3 autoclavings with 5 ml of water at 128 C for 3 hours each (*Neuman & Logan* 1950) Between each autoclaving the gelatine containing supernatant was collected after centrifugation at room temperature for 10 minutes at 600 × g

In all the radioactive samples the activities of ^{14}C-OH-proline were determined in salt soluble and insoluble collagen as well as in the non-gelatinized residue of the aortae. The ^{14}C-OH-proline isolation and measurement were performed as described by *Juva & Prockop* (1966) The vials were counted for 30 minutes in a scintillation spectrometer (Packard, model 3385) The efficiency of the ^{14}C counting was 70 per cent and the background was 20 cpm. The activities of ^{14}C-OH-proline in the aorta samples on the 42nd day were less than 2 times the background and are not included in the results.

The analyses mentioned below were only performed on samples of skin and granulation tissue due to the small amount of tissue which was obtainable from the aorta

The degree of collagen cross-linking *in vivo* was determined from the increase in ^{14}C-OH-proline activity in insoluble collagen after incubation of skin samples and granulation tissue at 37° C for 17 hours in 0.5 M NaCl in 0 02 M Tris-HCl, pH 7 4 followed by 3 salt extractions at 4 C overnight A possible influence of the sponge material and granulation tissue *per se* on the results was ruled out by adding non-radioactive sponge and granulation tissue to some radioactive skin samples during the incubation at 37 C No effect of the sponge and granulation tissue was detected on the ^{14}C-OH proline activities in salt soluble and insoluble collagen of the skin

The amount of free OH proline was determined in the non-hydrolyzed water extract of samples of skin and granulation tissue

The non-lyophilized granulomas and samples of fresh skin from the lumbar regions were minced

AORTA
(lyophilized and ground)

Total OH-proline determined in 2 mg samples after hydrolysis.

10 mg samples extracted 4 times overnight with 5 ml of 0.5 M NaCl + 0.02 M Tris-HCl pH 7 4

Residue autoclaved 3 times with 5 ml of water for 3 hours at 128 C. Centrifuged at 600 × g for 10 min. between each autoclaving.

Salt soluble collagen determined as the OH-proline in the combined supernatants after hydrolysis.

Non-gelatinized residue containing the elastin.

Combined supernatants containing gelatine = *insoluble collagen*

SKIN AND GRANULOMAS
A. Lyophilized and ground tissue

Total OH proline determined in 10 mg samples after hydrolysis.

Free OH proline determined in the 5 ml water extract of 50 mg samples.

Salt soluble collagen 4° determined from 50 mg samples as the OH-proline in the combined and hydrolyzed supernatants after 4 extractions overnight with 5 ml of 0.5 M NaCl + 0.02 M Tris-HCl pH 7 4 at 4 C.

Residue = *insoluble collagen 4°*

Salt soluble collagen "37°" determined from 50 mg samples as the OH-proline in the combined and hydrolyzed supernatants after 1 extraction at 37 C and 3 extractions at 4 C overnight with 5 ml of 0.5 M NaCl + 0.02 M Tris-HCl, pH 7 4

Residue = *insoluble collagen "37°"*

B Fresh tissue minced with scissors, and extracted 3 times overnight with 5 ml of 0.5 M acetic acid at pH 3 4

Residue discarded.

In the combined supernatants the collagen was precipitated with 15 per cent NaCl, dissolved in 0.5 M acetic acid, and reprecipitated twice with 10 per cent NaCl Collagen was finally precipitated by dialysis against 0 1 M disodium phosphate and water collected by centrifugation and lyophilized.

Fig 1 Flow sheet of the preparation of collagen fractions from rat aorta, skin and granuloma. (Hydrolysis carried out with 6 M HCl at 136 C for 6 hours. If not stated otherwise, the extractions were carried out at 4 C, and the samples centrifuged in refrigerated centrifuges at 45 000 × g for 30 minutes between each extraction)

with scissors in 0.5 M acetic acid 4 C, pH 3 4 immediately after the death of the rats, in order to inhibit any enzymatic processes, like procollagen peptidase. One drop of octanol was added as preservative. The collagen was extracted 3 times overnight and purified by 3 precipitations with 15 per cent and 10 per cent sodium chloride followed by dialysis against 0 1 M disodium phosphate and water (*Heikkinen* 1968) The acid extracted and purified collagen, which contains salt soluble as well as acid soluble collagen, was used for determinations of reactive aldehydes and for measurements of the ratio between single peptide chains (alpha chains) and double peptide chains (beta chains) The aldehyde content was determined according to the method of *Paz et al.* (1965) The samples were gelatinized for 10 minutes at 60 C in 0 1 M acetic acid at pH 3 4 and acetaldehyde was used as a standard. The alpha and beta chains in the purified collagen were separated by dodecylsulphate-polyacrylamide electrophoresis (*Furthmayr & Timpl* 1971) and the alpha/beta ratio determined by densitometric scanning of the gels on a Chromoscan 200 The alpha and beta bands in the gels were identified by the detection of OH-proline in the bands and by molecular weight determinations according to *Furthmayr & Timpl* (1971)

Statistical Methods

The effect of the sponge implantation and of the duration of the experiment on the variables in aorta, skin, and blood, was evaluated by a two-way analysis of variance, while the effect of time

TABLE 1 *Body Weights of Rats and Dry Weights of Samples and Percentage of Water in Aortas Skin and Granulomas*

Duration of the experiment in days		Body weight g	Aorta		Skin (7 cm^2)		Granuloma					
							mg DW			% H_2O		
			mg DW	% H_2O	mg DW	% H_2O	Mean	SEM	n	Mean	SEM	n
0	— op.	145	13 8	319	251	196	—	—	—	—	—	—
4	— op.	178	16.3	326	289	179	—	—	—	—	—	—
	+ op.	171	15 1	249	274	175	144	6	6	1273	33	6
8	— op	204	20.3	276	350	175	—	—	—	—	—	6
	+ op.	202	20 7	272	265	169	269	7	6	827	28	
14	— op.	256	25.5	240	358	140	—	—	—	—	—	—
	+ op	251	24 4	247	323	139	286	7	12	661	10	12
22	— op	298	28.3	252	329	160	—	—	—	—	—	—
	+ op.	290	26.2	262	326	164	323	28	6	590	19	6
33	— op	342	31 1	227	456	134	—	—	—	—	—	—
	+ op.	350	31 0	228	456	134	330	15	6	313	18	6
[illegible]	— op.	385	33.5	251	500	123	—	—	—	—	—	—
	+ op	382	31 6	253	470	114	333	8	11	423	26	11
n		6	6	6	6	6						
S.D		18	2.3	32	43	24						
P-values												
Operation		n.s.	<0.05	n.s.	n.s.	n.s.						
Time		<0 01	<0 01	<0 01	<0.01	<0.01						
Interaction		n.s.	n	<0 05	<0 01	n.s.						

op. = implantation of subcutaneous sponges on the day 0
SEM = standard error of mean. n = number of animals in each group.
DW = dry weight. % H O = percentage of water per dry weight
S.D = standard deviation within the groups.
n.s. = not statistically significant
The body weights and the values from aorta and skin are given as means, and tested with a two-way analysis of variance.

TABLE 1. *Total Content of Collagen in Aorta Skin and Granulomas of Rat and the Content of Non gelatinized Tissue (Elastin) in Aorta*

Duration of the experiment in days		Aorta µg OH-proline		Skin mg OH proline per 7 cm²	Granuloma mg OH-proline		
		Elastin	Collagen		Mean	SEM	n
0	— op	109	278	15 1	–	–	–
4	— op.	119	251	–	–	–	–
	+ op.	127	287	–	1.81	0.38	4
8	— op.	156	383	17 1	–	–	–
	+ op.	126	437	13.3	3.07	0.35	6
14	— op.	175	536	20.3	–	–	–
	+ op.	183	563	20.3	4.86	0.48	12
22	— op	15	764	30.9	–	–	–
	+ op.	210	689	28.2	7.56	1 11	6
33	— op.	281	975	1.8	–	–	–
	+ op.	251	889	25 2	7 16	0.55	6
42	— op.	225	821	31.9	–	–	–
	+ op.	214	783	29 4	7.91	0.70	10
n		6	6	6			
SD		30	145	3 4			
P-values							
Operation		n.s.	n.s.	n.s.			
Time		<0.01	<0.01	<0.01			
Interaction		n s.	n.s.	n.s			

op = implantation of subcutaneous sponges on th day 0
SEM = standard error of mean n = number of animals in each group.
SD = standard deviation within the groups
n = not statistically significant.
– = missing values due to technical error
The values from aorta and skin are given as means, and tested with a two-way analysis of variance.

on granulation tissue was tested by a linear regression analysis. The results from the radioactiv samples, hich only include 2 or 3 time intervals, were evaluated by use of student t test Only P-values less than 0.05 were regarded as statistically significant.

RESULTS

A Tissue Difference

The dry weights of the granulomas (Table 1) and their content of collagen (Table 2) increased during the first 22 days, but were unchanged hereafter The water percentage of the granulomas decreased during the entire 42 days, but was all the time much higher than in aorta and skin (Table 1) The collagen synthesis, expressed as the total activity of ^{14}C-OH-proline (Table 3) was unchanged from the 14th to the 42nd day in the granulomas and in skin. In aorta the collagen synthesis increased from day 0 to day 14 while the ^{14}C-OH proline uptake in the non-gelatinized residue was unchanged. The specific activity of ^{14}C-OH-proline in the collagen of 7 animals on the 14th day was 11.2 dpm per µg OH proline in granulation tissue (SEM = standard error of mean = 1 6) 4.3 (SEM = 0.4) in skin, and 3.6 (SEM = 0 in aorta. The values were significantl in granulation tissue than in skin

TABLE 3 *Total ^{14}C-Hydroxyproline Activities in Aortae Skin and Granulomas of Rats 4 Hours after ^{14}C-proline Administration in Vivo*

Duration of the experiment in days		Aorta: Elastin Mean	Elastin SEM	dpm n	Collagen Mean	Collagen SEM	n	Skin dpm × 10^{-3} per 7 cm² Mean	SEM	n	Granuloma dpm × 10^{-3} Mean	SEM	n
0	— op	367	37	9	1339	75	11	88	17	4	–	–	–
14	— op	361	41	11	2307	223	8	96	12	12	–	–	–
	+ op.	313	40	8	1995	248	9	81	12	11	31	10	10
42	— op.	–	–	–	–	–	–	79	11	9	–	–	
	+ op.	–	–	–	–	–	–	76	14	10	58	6	9

op = implantation of subcutaneous sponges on the day 0
SEM = standard error of mean. n = number of animals in each group
The values are compared with student s t-test.
^{14}C-OH proline activity per aorta was increased on the 14th day in collagen ($P<0.001$) but not in the non-gelatinized tissue (elastin) when compared with day 0 No influence of time was found on the ^{14}C-OH-proline activity per square unit of skin or per granuloma. No influence of the operation was found on the values from aorta and skin

($P<0.01$) while no statistically significant difference was detectable between skin and aorta. The rate of collagen cross-linking expressed as the per cent of ^{14}C-OH proline which could not be extracted with 0.5 M NaCl after 4 hours of labelling *in vivo* was double as high in the granulomas and aortae than in skin (Table 4) Incubation at 37 C *in vitro* did not change the distribution of ^{14}C-OH proline between salt soluble and insoluble collagen in the granulation tissue whereas in skin, incubation at 37 C, doubled the ^{14}C-OH proline activity in insoluble collagen (Table 4)

The total amount of free OH proline in the granulomas increased up to day 33 (Table 5) The free OH proline, as per cent of total OH-proline was about 5 times as high in granulation tissue than in skin but decreased in both tissues during the 42 days of experiment. Salt soluble collagen decreased in all the tissues during the experiment, but was of the same magnitude in granulation tissue and in skin, and higher in these tissues than in the aorta (Table 5)

The alpha/beta ratio and the aldehyde content in purified collagen was higher in granulation tissue than in skin. A linear regression analysis showed an increase in the alpha/beta ratio in collagen from the granulomas during the 42 days ($P<0.05$) in contrast to the fall in the ratio in the skin (Table 6) Aldehyde determinations were only carried out on 33 and 42 day old granulomas due to the small amounts of purified collagen which were obtainable from the younger granulomas.

B Effect of Sponge Implantation

The implantation of subcutaneous sponges had no influence on the normal weight gain of the rats (Table 1) However the dry weights of aortae and skin samples were somewhat lower in the operated animals than in the non-operated (Table 1) The total content of collagen in the aortae and skin samples, and the non-gelatinized residue in the aortae (Table 2) were not decreased in the operated animals in contrast to the sample weights. No influence of the operation was found on the ^{14}C-OH proline uptake in the tissues (Table 3) or on the distribution of ^{14}C-OH-proline between salt soluble and salt insoluble collagen (Table 4) Neither did the sponge implantation affect salt soluble and

TABLE 4 *Per Cent of ¹⁴C-Hydroxyproline F and in Salt Insoluble Collagen in Aort Skin and G anulomas / Rats*

Duration of the experiment in days	Aorta 4** Mean	Aorta 4** SEM	Aorta 4** n	Skin 4** Mean	Skin 4** SEM	Skin 4** n	Skin P-values	Skin "37** Mean	Skin "37** SEM	Skin "37** n	Granuloma 4 Mean	Granuloma 4 SEM	Granuloma 4 n	Granuloma P-values	Granuloma "37** Mean	Granuloma "37** SEM	Granuloma "37** n
0 — op.	66	4	11	20	5	7	<0.001	67	4	7	–	–	–		–	–	–
14 — op.	67	2	8	33	3	10	<0.001	76	6	10	–	–	–		–	–	–
14 + op	61	2	9	37	5	8	<0.001	77	4	8	71	3	10	n.s.	73	3	10
42 — op.	–	–	–	24	3	6	<0.001	58	7	6	–	–	–		–	–	–
42 + op.	–	–	–	29	3	9	<0.001	63	5	9	64	3	9	n.s.	64	3	9

op. = Implantation of subcutaneous sponges on the day 0. SEM = standard error of mean. n = number of animals. n.s. = not statistically significant.
P-values derived by student's t test.
4** = per cent of total ¹⁴C-OH-proline found in insoluble collagen after 4 extractions at 4 C with 0.5 M NaCl.
"37** = per cent of total ¹⁴C-OH-proline found in insoluble collagen after 1 extraction at 37 C and 3 extractions at 4 C with 0.5 M NaCl.
No statistically significant influence of sponge implantation was found on the values from aorta and skin.

TABLE 5 *Salt Soluble Collagen as Per Cent of Total Collagen in Aortae Skin and Granulomas and Free Hydroxyproline as Per Cent of Total Collagen in Skin and Granulomas and Total Amount of Free Hydroxyproline per Granuloma of Rats*

Duration of the experiment in days		Salt soluble collagen per cent					Free OH-proline per cent				Free OH proline µg per granuloma		
		Aorta	Skin	Granuloma			Skin	Granuloma					
				Mean	SEM	n		Mean	SEM	n	Mean	SEM	n
0	— op.	1.52	6.13	–	–	–	0.22	–	–	–	–	–	–
4	— op.	2.61	2.65	–	–	–	0.16	–	–	–	–	–	–
	+ op.	3.07	2.33	6.36	0.31	5	0.12	1 26	0 46	4	24 1	1 1	6
8	— op.	1 72	1.91	–	–	–	0 19	–	–	–	–	–	–
	+ op.	1.60	4.08	3.56	0.24	6	0.17	0.90	0 15	8	37.3	0 7	6
14	— op.	0.96	2 13	–	–	–	0.08	–	–	–	–	–	–
	+ op.	1.38	2.22	2 70	0.21	12	0.09	0 61	0 05	12	35 4	1 0	12
22	— op.	1.58	2.93	–	–	–	0.10	–	–	–	–	–	–
	+ op.	1.50	2.20	2.33	0.49	5	0.10	0 46	0.04	6	43 0	4.6	6
33	— op.	0.93	2.90	–	–	–	0.09	–	–	–	–	–	–
	+ op.	0.84	2.94	2.29	0 76	6	0.11	0 53	0.02	6	47.5	1.5	6
42	— op.	0.74	1.81	–	–	–	0.06	–	–	–	–	–	–
	+ op.	0.90	1.51	2.64	0.50	11	0.06	0 41	0.03	9	37 1	1.3	11
n		6	6				6						
S.D		0 77	1.37				0.03						
P-values:													
Operation		n.s.	n.s.				n.s.						
Time		<0 01	<0.01				<0.01						
Interaction		n.s.	n.s.				n.s.						

op. = implantation of subcutaneous sponges on the day 0.
SEM = standard error of mean. n = number of animals in each group.
S.D = standard deviation within the groups.
n.s. = not statistically significant.
The values from aorta and skin are given as means, and tested with a two-way analysis of variance.

TABLE 6. *Alpha/beta Ratio and Aldehyde Content in Acid Extracted and Purified Collagen from Rat Skin and Granuloma*

Duration of the experiment in days		Alpha/beta ratio				Aldehyde/collagen (moles/mole)			
		Skin	Granuloma			Skin	Granuloma		
		Mean	Mean	SEM	n	Mean	Mean	SEM	n
0	— op.	1.30	–	–	–	2.44	–	–	–
4	— op.	1.16	–	–	–	2.89	–	–	–
	+ op.	1.29	2.68	0.20	2	2.42	–	–	–
8	— op.	1.32	–	–	–	2.23	–	–	–
	+ op.	1.40	3.17	0.34	5	2.24	–	–	–
14	— op.	1.29	–	–	–	2.09	–	–	–
	+ op.	1.27	2.39	0.11	2	2.29	–	–	–
22	— op.	1.27	–	–	–	1.79	–	–	–
	+ op.	1.23	3.48	0.33	3	1.60	–	–	–
33	— op.	1.24	–	–	–	2.04	–	–	–
	+ op.	1.28	3.37	0.27	5	1.92	3.24	0.41	4
42	— op.	1.15	–	–	–	2.06	–	–	–
	+ op.	1.13	3.63	0.26	11	1.65	2.63	0.73	11
n		5				5			
S.D		0.10				0.53			
P-values									
Operation		n.s.				n.s.			
Time		<0.01				<0.01			
Interaction		n.s.				n.s.			

op. = implantation of subcutaneous sponges on the day 0
SEM = standard error of mean n = number of animals in each group.
S.D = standard deviation within the groups.
n.s. = not statistically significant.
The values from the skin are tested with a two-way analysis of variance.

free OH-proline (Table 5) alpha/beta ratio and aldehyde content (Table 6) of the skin.

The hemoglobin concentration in the blood increased during the 42 days from 11.6 to 14.6 g per 100 ml (S.D = standard deviation within the groups = 0.5 $P<0.01$) but was unaffected by the sponge implantation. Also the number of leucocytes in the blood, which increased during the experiment from 6300 to 12100 per µl blood (S.D = 1800 $P<0.01$) was not influenced by the operation. However the percentage of granulocytes, which increased during the experiment from 18 to 29 per cent (S.D = 6, $P<0.01$) was higher in the operated animals than in the non-operated ($P<0.01$)

DISCUSSION

The problem of quantitative isolation of granulation tissue without contamination from surrounding tissue, has commonly been solved by subcutaneous implantation of sponges into which granulation tissue grows. The most frequently used materials have been polyvinyl sponges (*Boucek & Noble* 1955 *Edwards et al.* 1957 *Boucek et al.* 1959 *Woessner & Boucek* 1961 *Bole & Robinson* 1962, *Bailey et al.* 1973) and viscose cellulose sponges (*Viljanto & Kulonen* 1962 *Viljanto* 1964 *Akonen* 1968, *Lehtonen* 1968, *Rantanen* 1973) *Berg* (1955) has shown that plastic sponges are absorbed after several months

of subcutaneous implantation. Most authors agree that apart from the presence of giant cells, the histological and biochemical development of granulation tissue in subcutaneous sponges are similar to that of wound granulation tissue, and independent of the chemical nature of the sponges.

The unchanged collagen synthesis per granuloma from the 14th to the 42nd day (Table 3) in spite of a decreased collagen accumulation after the 22nd day (Table 2) may imply that collagen degradation increases in older granulomas (*Boucek et al.* 1959). This is in accordance with the increased activity of degradative enzymes in granulomas after 3 weeks (*Woessner & Boucek* 1961) and the increase in collagen turn over in skin wounds after 3 weeks of healing (*Madden & Peacock* 1971). The high percentage of salt insoluble ^{14}C-OH-proline in granulation tissue and aorta compared with skin (Table 4) indicates that the formation of salt insoluble collagen from soluble collagen in granulation tissue and aorta is increased compared with skin. An increased insolubilization of collagen may be due to collagen interactions with other components of the connective tissue, e.g. the proteoglycans (*Öbrink* 1973, *Scott et al.* 1975) or it may be caused by an increased formation of neutral salt stable intermolecular cross-links in the collagen. The formation of cross-links in collagen depends on the formation of aldehydes by deamination of lysine and OH lysine (*Bornstein et al.* 1966). The formation of aldehydes and cross-links in rat skin collagen after incubation at 37° C *in vitro* has been shown by *Desmukh et al.* (1971). The unchanged distribution of ^{14}C-OH-proline between salt soluble and salt insoluble collagen in granulation tissue after incubation at 37° C (Table 4), in contrast to skin, may reflect that collagen from granulation tissue already *in vivo* has formed the maximal amount of salt stable intermolecular cross-links. This presupposes a faster rate of aldehyde formation in granulation tissue and is supported by the higher aldehyde content in the acid extracted collagen from the granulomas than in skin (Table 6).

The higher specific activity of ^{14}C-OH proline in granulation tissue than in skin, indicates a relatively higher rate of collagen synthesis in the granulomas. However the salt soluble collagen which is mainly derived from *de novo* synthesis of collagen (*Jackson & Bentley* 1960, *Laitinen* 1967) was of the same magnitude in the granulation tissue as in skin (Table 5). This may be explained by the higher rate of cross-linking of collagen in granulation tissue than in skin, as discussed above. Similar assumptions may explain the lower percentage of salt soluble collagen in the aorta as compared with skin. The specific activities of ^{14}C-OH-proline in aorta and skin *did not differ statistically significantly* from each other, but the cross-linking of collagen when measured as insoluble ^{14}C-OH proline (Table 4) was higher in aorta than in skin. The decrease in salt soluble collagen in the tissues by time is generally correlated to the decreasing growth rate in older animals (*Gross* 1958, *Wirtschafter & Bentley* 1962).

The free OH-proline in the tissues originate from collagen degradation (*Prockop & Kivirikko* 1968). The products of collagen degradation also include small OH-proline containing peptides which are not measured when free OH-proline is analyzed. Furthermore, the amount of free OH proline in the tissues depend on the removal of the OH proline via excretion in the urine or via further degradation to carbon dioxide and urea. Consequently the amount of free OH proline in a given tissue does not necessarily reflect the true rate of collagen degradation. However, the increase in the total amount of free OH proline in the older granulomas (Table 5) is compatible with an increased collagen degradation in the older granulomas. The high percentage of free OH proline in granulation tissue, as compared with skin, may reflect a relatively higher collagen degradation in granulation tissue than in skin.

The ratio of alpha chains to beta chains in collagen reflects the formation and stability of the intramolecular cross-links in the collagen molecule. The alpha/beta ratio in rat

skin and rat tail tendon has been reported to decrease with age (*Heikkinen & Kulonen* 1964). This is in accordance with the results from rat skin in the present investigation (Table 6). However, in collagen from the granulation tissue the alpha/beta ratio increases during the 42 days of experiment. This increase in the older granulomas may be secondary to an increased collagen degradation rather than to an increased *de novo* production of alpha chains, as the rate of collagen synthesis is unchanged from the 14th to the 42nd day in the granulomas (Table 3). The degradation of collagen has been reviewed by *Harris & Krane* (1974). A specific collagenase, which breaks the collagen triple-helix across, is present in several tissues, but also collagenolytic cathepsins, which will cleave the interchain cross-links in collagen yielding alpha chains, may be of physiological importance (*Etherington* 1974).

The subcutaneous sponge implantation increased the number of granulocytes in the blood and decreased the weight of the aortae and skin samples. However, the collagen variables were not influenced by the sponge implantation to an extent which was statistically significant. This is in contrast to the frequent reported distant collagen response to wound healing, which has been reviewed by *Viidik et al.* (1972). However, even though the effect of the sponge implantation on the tissues and blood in the present investigation was small, the possible effect of an experimental trauma on the analyses must always be considered.

In conclusion, the rate of collagen crosslinking was increased in granulation tissue and aortae as compared with skin. The degradation of collagen increased in the older granulomas and was accompanied with an increase in alpha/beta ratio in acid extracted collagen. The implantation of subcutaneous sponges did not influence the collagen analyses. It decreased the weight of aorta and skin and increased the number of granulocytes in the blood.

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DIFFERENT EFFECTS ON RENAL AND SUBMAXILLARY RENIN RELEASE AFTER BLOCKADE OF THE RENIN SYSTEM IN MICE

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Bing, J & Poulsen, K. Different effects on renal and submaxillary renin release after blockade of the renin system in mice. Acta path. microbiol. scand. Sect. A, 83 733–736 1975

Inhibition of the angiotensin I converting enzyme with SQ 20,881 results in a rapid marked increase in plasma renin concentration in mice. The maximum effect is short lasting, but the values are still elevated 2 hours after the injection, the time course being similar to that previously found in rats. The relative increase is the same in normal and sialo-adenectomized mice. The plasma renin concentration of nephrectomized mice with their submaxillary glands intact is, however totally uninfluenced by injection of the blocker. This finding indicates that renin release from the submaxillary glands is not controlled by the plasma angiotensin II concentration as is that of the renin release from the kidneys.

Key words Renin release renin system blockade.

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A renin-like pressor principle in the submaxillary gland was first demonstrated in albino mice by *Werle et al* (1957) The location and physiology of this submaxillary iso-renin has since been studied (for literature see *Page & McCubbin* 1968 *and Bing & Poulsen* 1971) The present study aims at elucidating whe ber or not blockade of the renin system plasma stir lates renin to release fr the ubmaxillary glands of mice as it does m kidneys (for literature see *Davis et al* 19

MA ERI ND METHODS

Animals [illegible] mice of the Danish State Ser[illegible] weighing about 35 to 60 g. The mice were *conscious* a catheter had been inserted in the femoral artery during short-term ether anaesthesia. After the operation the mice were placed in restraining cages in the form of perforated polyethylene tubes. In order to obtain basal conditions after the anaesthesia and the operation the mice were kept for 3 hours in these cages before the start of the experiments. During this time clotting in the catheters was avoided by infusion of 0 12 ml/h of 5 per cent glucose-solution containing 50 I U heparin/ml.

Sialo-adenectomy was performed either 2–4 days or more than 10 days before the experiment, and *nephrectomy* either about 18 hours before or at the start of the experiments. These differences in time did not play any role for the results.

Blockade of the renin system was obtained by intraarterial injection of 2 mg/kg of an angiotensin I converting enzyme inhibitor the synthetic nonapeptide > Glu-Trp-Pro-Arg Pro-Glu-Ile-Pro-Pro, designated *SQ 20,881* (Squibb Corp Prince

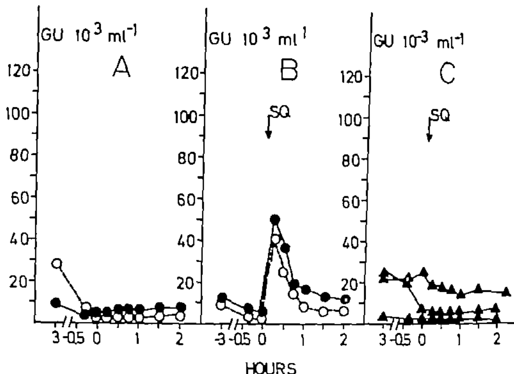


Fig 1 Effect of SQ 20.881 on plasma enin concentration with time in normal sialo-adenectomized and nephrectomized mice A Controls. Mean plasma renin concentration with time in 5 normal (●) and 3 sialo-adenectomized (○) mice immediately after the anaesthesia and operation (time + 3) about 2½ and 3 hours later and at different times in the following two hours *B. Effect of 2 mg/kg SQ 20.881 on* mean plasma renin concentration with time in 9 normal (●) and 11 sialo-adenectomized (○) mice. *C Lack of effect of 2 mg/kg SQ 20.881* on plasma renin in 3 individual *nephrectomized* (▲) mice.

Plasma renin concentration was determined in plasma from about 20 µl samples of blood drawn from the femoral artery using the capture radioimmunoassay for angiotensin I as described by *Poulsen & J rgensen* (1974) Th concentration was expressed in Goldblatt units (G U) × 10^{-3} × ml by comparison with the tandard hog renin preparation obtained from D vision for Biological Standards, Holly Hill London, donated by Dr *Haas* Cleveland, U.S.A. *Renal and submaxillary enin content* of kidneys and submaxillary glands was determined with the same method after th tissues had been frozen and thawed 3 times and then homogenized at zero degrees in a 0.02 M pyrophosphate buffer pH 5.3 by using a Potter El ehjem homogenizer The samples were left for about 18 h at 4 C before centrifugation.

RESULTS

I Renin Content of Kidneys and Submaxillary Glands

The mean *renal renin concentration* in 6 mice weighing about 35 g was 77 G U per g (range 50–100) which with a mean weight of the two kidneys of 500 mg gives a mean total renal renin content of 40 G U In the *submaxillary glands* the corresponding values were a mean concentration of 21.500 G.U per g (range 8.000–59 000) a mean total weight of the two glands of 105 mg and thus a total submaxillary renin content of 2.300 G U This means that the submaxillary glands contained 60-fold the content of the kidneys.

II Plasma Renin Con ntration with Time after a Short Ether Anaesthesia and Placement of Catheter in One Femoral Artery (Controls without Blockade of the Renin System)

The variation of plasma renin concentration with time was studied in 8 mice (5 normal and 3 sialo-adenectomized) which after a short ether anaesthesia and placement of

catheter in one femoral artery were continuously infused with the small volume glucose solution containing heparin, which was also used in the following experiments with injection of blocker Fig 1 A shows that anaesthesia and operation result in an increase in plasma renin concentration, which is most pronounced in the sialo-adenectomized. Basal renin concentration is obtained 3 hours after the anaesthesia (time zero) and is thereafter constant or slightly increasing throughout the experimental period.

III Effect on Plasma Renin Concentration of Intraarterial Infusion of the Angiotensin I Converting Enzyme Inhibitor SQ 20.881

A. Normal mice In 9 conscious mice, the injection of a dose of 2 mg/kg b.w of SQ 20.881 was followed by a rapid, marked increase in renin concentration. In about 15 minutes it rose about 13-fold (range 7–23) from an average of 4.3 GU $\times 10^{-4}$ (range 1–6.6) to an average of 50 GU $\times 10^{-4}$ (range 14–91) The effect was, as is seen in Fig 1 B short-lasting. The values declined already after 30 minutes, but still 2 hours after the injection the renin concentration was about 2.5-fold higher than the value found before the blocker was injected.

B. Sialo-adenectomized mice In 11 sialo-adenectomized mice the injection of the same dose of SQ (2 mg/kg b.w) was followed by quite similar changes in plasma renin concentration as found in normal mice. The only difference was that in the sialo-adenectomized the values were a little lower throughout the experiment At the time of the injection (marked 0 in Fig 1 B) it was in average 3.2 GU $\times 10^{-4}$ [illegible] 1.9–5.0) 15 minutes after the injectio[illegible] average was 41 GU $\times 10^{-4}$ (range 20–1[illegible] The relative changes were thus identi[illegible] normal and sialo-adenectomized mice.

C Nephrectom[illegible]d mice In 9 nephrectomized mice the re[illegible] concentration showed very marked ind[illegible]idual differences both in the plasma taken [illegible] the end of the operation and at the time when the blocker SQ 20.881 was given. Both high and low plasma renin levels were found as well in 18-hour nephrectomized as in mice nephrectomized at the start of the experiment. In none of the animals did a blockade of the renin system result in any increase in the plasma renin concentration. The values remained the same or were a little lower than that found at the time of injection. The mean values varied from 90 to 70 per cent of the value at time zero (Fig 1 C)

DISCUSSION

Differences between renal and submaxillary renin have previously been shown by *Turrian* (1960) and *Oliver & Gross* (1967) who found that chronic sodium loading, even when combined with DOCA administration, results in pronounced decrease in renal renin content, but does not influence the content in the submaxillary glands. *Menzie et al.* (1974) have further shown that the sympathetic nervous system stimulates renin release at the two sites by different mechanisms, namely by way of beta-adrenergic receptors in the kidney and via alpha-adrenergic receptors in the submaxillary glands. The individual variation and sometimes high level of plasma renin in the nephrectomized mice in the present study might be due to different degrees of stimulation of the receptors in the submaxillary gland, caused by anaesthesia and operation.

The present study has shown that blockade of the renin system with the angiotensin I converting enzyme inhibitor SQ 20.881 causes a marked and rapid, shortlasting and identical release of renin in normal and sialo-adenectomized mice. The time course and degree of the changes in plasma renin after blockade of the renin system is very similar to that obtained in rats (*Bing & Poulsen* 1975)

If the mice were nephrectomized prior to the blockade no submaxillary renin was released to the blood. This demonstrates that the plasma angiotensin II concentration is not a regulator of submaxillary renin as it is for renal renin.

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BRIEF REPORTS

VARIATION IN DISTRIBUTION OF ANTRAL GASTRIN CELLS

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Aames, S. & Johansen, A. Variation in distribution of antral gastrin cells. Acta path. microbiol. scand. Sect. A, 83 737–738, 1975.

Using immunoperoxidase technique a considerable variation in the number of antral gastrin cells was demonstrated in patients with atrophic gastritis. This variation must be taken in consideration when the number of gastrin cells is to be estimated on the basis of gastric biopsies.

Key words Antral gastrin cells trophic gastritis variation.

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After the isolation and chemical characterization of human gastrin (5) the first successful application of an immunofluorescence technique for localization of gastrin in pyloric mucosa was made by *McGuigan* in 1968 (7) Demonstration by peroxidase labelled antibodies as described by *Avrameas* (1) has also been used and found just as specific as immunofluorescence (8)

The gastrin producing cells (g-cells) in the pyloric gland area has been found to be localized in the mid zone of the glands, the base of the cell along the basal membrane. They are round, pear shaped or of pyramidal form. Ultrastructural studies reveal numerous gastrin granules in the basal part of the cell (3)

According to the literature the number of g-cells is ariable in different gastric diseases and the number has been noticed to be increased in cases of hyperacidity and pernicious anaemia as well as in different endocrine disorders (3 4 9)

It should be expected that ariation in the number of g-cell had to be compared w th the normal state, but as noted by *Lam & Sircus* (1974) data on the range and number of g-cells in the normal human antrum have not yet been published. Furthermore the influence of histological changes in the pyloric mucosa, for example trophic gastritis, on the number and distribution of g-cells has not been described.

Since estimation of the number could influence the diagnosis and treatment of gastric diseases it is of interest to investigate whether gastric biopsy is a method sufficient for an evaluation of the total g-cell mass in the antrum.

Such studies has recently been published by *Polak et al.* (1975) who conclude that even a small number of biopsy samples was representativ of this evaluation.

In our department we have studied the variation of g-cells by an immunoperoxidase technique implying the use of antibodies raised against synthetic human gastrin I (11) The material include operation specimens and endoscopic biopsies from 65 patients with different gastric disorders. Our experience is that the number of g-cells in the pyloric mucosa is arying considerably

This is illustrated by the four Figures (Fig 1–4) Each of them is representative of *one* of four biopsies taken from a small area of the antral mucosa in a patient with an ordinary trophic gastritis. The number of cells is ary g from 1 to 165 but each illustration represents or ly about one third of the area in an endoscopic biopsy

I our opinion this means that the uncertainty involved in estimating the g-cell mass from small number of biopsies is considerable First, the variation of the number of the cells at different stages of gastritis must be fully known

To obtain this knowledge t will be necessary to use a method of exact quantitation. The number of cells in well-defined areas of the mucosa must

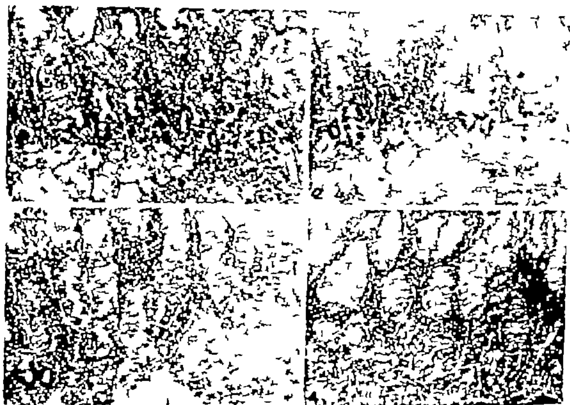


Fig 1–4 Variation in number of g-cells demonstrated by four neighbouring mucosa samples from the same patient.

Fig 1 Histologically normal mucosa area with numerous g-cells. (× 130)

Fig 2 Neighbouring area of the mucosa still normal, with considerably fewer g-cells. (× 130)

Fig 3 Severe pyloric gastritis with focal distribution of g-cells. (× 130)

Fig 4 Severe pyloric gastritis with only one g-cell. (× 130)

onstantly be related to the histological changes in lifferent well-controlled groups. Furthermore, if he level of circulating gastrin and the concentraion of immunoreactive gastrin in the pyloric ancosa are related to this information it might be ossible to obtain knowledge from a pyloric biopsy ust as informative as the knowledge about acid ecretion to be obtained from a corporic biopsy 2). Such a method is being developed in our aboratory by means of the immunoperoxidase echnique.

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SPONTANEOUS TOXIC NEPHROPATHY IN POULTRY ASSOCIATED WITH OCHRATOXIN A

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Elling, F., Hald, B., Jacobsen, Chr & Krogh, P Spontaneous toxic nephropathy in poultry associated with ochratoxin A. Acta path. microbiol scand. Sect. A, 83: 739–741 1975

At poultry slaughterhouse 14 birds with macroscopic renal changes were collected, and the kidneys were examined histologically and the muscular tissue was analysed for ochratoxin A residues. Out of 14 birds 5 birds had ochratoxin A residues ranging from 4.3 to 29.2 μg/kg. In 4 of these birds a toxic nephropathy was found characterised by atrophy and degeneration of proximal and distal tubules and interstitial fibrosis. The possibility of birds with ochratoxin A residues being presented for human consumption is discussed.

Key word Ochratoxin A poultry toxic nephropathy residues of ochratoxin A.

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Ochratoxin A, a nephrotoxic secondary metabolite of several fungal species belonging to Aspergillus and Penicillium, has been found as a natural contaminant of plant products especially cereals. This mycotoxin is causally associated with porcine nephropathy (4) a spontaneously occurring disease in pigs (1) which has been experimentally reproduced by the fee ing of ochratoxin A—contaminated feed (2) As ot only pigs but also poultry may be exposed   ochratoxin A—contaminated cereals, an in estigation was conducted in order to elucidat th possible occurrence of mycotoxic nephropa h   poultry similar to mycotoxic porcine nephrop…

Material and Method

During the meat inspection t   poultry slaughterhouse (Danpo, Vamdrup J tland) 14 birds condemned because   macroscopical renal changes, enlarged, pale kid eys, were collected.

One kidney lobe f m each bird was fixed in formalin, and sections were stained w th hematoxylin-eosin, ironhematoxylin-van Gieson, and PAS. Fifty grammes samples of thoracic muscular tissue were analysed for ochratoxin according to the procedure previously published (2) Positi findings of residues were confirmed by derivati formation. The procedure has a lower level of detection at ~3 μg/kg.

Results

In 5 of the 14 birds ochratoxin A   detected in amounts ranging from 4.3 to 2°   kg Morphological changes were constantly   the kidneys of 4 of these birds The   rul all appeared normal, but the proxim   d distal tubules were dilated with   rophic   lar cells In tw birds with 10.2 and 13 7 μg kg ochratoxin A the tubular epithel um showed degenerative changes in the form of balloo g   desquamation (Fig. 1) while the   the highest amount of ochratoxin A   μg/kg) demonstrated   marked diff se   roph   of the kidr   parenchyme and hyaline   were seen in



Fig. 1 Kidney from bird with 10.2 µg/kg ochratoxin A residue in the muscular tissue. Note the atrophic tubule (arrow) surrounded by fibrous tissue and the desquamation of epithelial cells (double arrow). H & E, × 400

Fig. 2 Kidney from bird with 13.7 µg/kg ochratoxin A residue in the muscular tissue. Note the interstitial fibrosis in the medullary tract and the thickened capsule surrounding the medullary tract. H & E, × 100.

of the atrophic tubules. No vascular lesions were found in any of the kidneys, but the amount of fibrous tissue was increased mainly in the medullary tracts and around the intralobular veins (Fig. 2). In the bird with the highest amount of ochratoxin A interstitial infiltration with eosinophils and mononuclear cells was encountered in the newly formed fibrous tissue.

No microscopical renal changes were found in one bird with 8.7 µg/kg ochratoxin A in the muscular tissue and in one bird with trace of ochratoxin A (less than 2 µg/kg).

Ochratoxin A was not detected in the remaining 8 of the 14 birds and while the kidneys of 6 of the 8 birds showed no microscopical kidney lesions, 2 birds demonstrated changes characterized by interstitial infiltration with macrophages, lymphocytes, and eosinophils. In these two birds the glomeruli appeared normal while in the proximal and distal tubules cell casts were frequently observed in the atrophic tubules. No vascular lesions were seen, but the interstitial infiltration was accompanied by fibrosis.

Discussion

The association between ochratoxin A residues in the muscular tissue and the renal structural changes seems to indicate that ochratoxin A is a disease determinant in toxic nephropathy in poultry as it is in pigs (2, 4). This hypothesis has been further strengthened by a recent feeding experiment with chickens using cereals naturally contaminated with ochratoxin A. Residues of ochratoxin A were detected in the muscular tissue, liver and kidney and generally similar renal structural changes were observed.

As it has been possible to induce renal damage with ochratoxin A in all mammals hitherto investigated, it is likely that this mycotoxin also may cause nephropathy in man. In this study the birds with ochratoxin residues were found among the

condemned birds. However the birds in the feeding experiment (3) containing ochratoxin A in muscular tissue liver and kidney would all have passed the meat inspection, because the pathological changes were only microscopical. Furthermore, not all poultry slaughterhouses have got veterinary inspection.

In conclusion, the present material represents spontaneously occurring toxic nephropathy in poultry associated with ochratoxin A, most likely feed borne, as indicated by residues in the muscular tissue. Our observations indicate additionally that birds containing ochratoxin A residues may very well be presented for human consumption.

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Acta path. microbiol scand. Sect. A, 83 741–743 1975

IDENTIFICATION OF CELLS WITH PANCREATIC-TYPE AND GUT TYPE GLUCAGON IMMUNOREACTIVITY IN THE HUMAN COLON

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Knudsen, J B., Holst, J J Asnæs, S. & Johansen, A. Identification of cells with pancreatic type and gut-type glucagon immunoreactivity in the human colon. Acta path. microbiol. scand. Sect A, 83 741 743 1975.

The existence of cells with pancreatic-type glucagon and gut-type glucagon immunoreactivity is for the first time demonstrated in the human colon using immunohistochemical techniques and specific antisera

Key word Glucagon human colon immunohistochemical techniques.

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The presence of glucagon in the alpha cells of pancreatic islets was established by immunofluorescence technique by *Baum et al* (1962) Structural evidence of glucagon producing cells in the intestinal mucosa was provided by *Orci* (1968) and cells containing glucagon-like immunoreactivity were identified by immunofluorescence technique and found to be localized to the gastrointestinal tract in the dog by *Polak et al* (1971) Intestinal glucagon-like bioactivity was described for the first time in 1948 (10) The present knowledge of the biological and immunological activity of gut glucagon was reviewed by *Falcona & Unger* (1974) Recently it was reported (11) that a substance indistinguishable from pancreatic glucagon, was located in the gastrointestinal mucosa of the pig and the dog Cells containing pancreatic-type glucagon and cells containing gut-type glucagon were observed in the pig, dog, and rat by *Larsson*

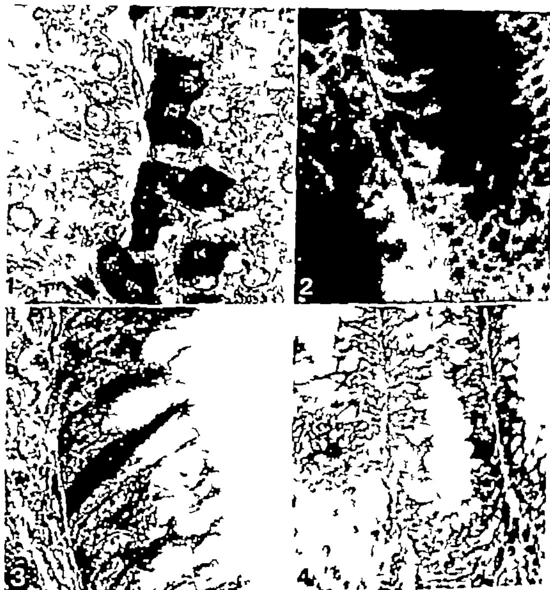


Fig 1 Alpha cells of the mouse pancreas demonstrated by the immunoperoxidase technique. (× 800)

Fig 2 and 4 Cells showing glucagon immunoreactivity in the human colon demonstrated by fluorescence technique and immunoperoxidase technique. (Fig 2 × 600 Fig 4 × 500)

Fig 3 Immunoperoxidase stained cell demonstrating glucagon immunoreactivity in the colon crypt. (× 850)

t al. (1975) using specific immunofluorescence technique. However no reports on the presence and possible type of glucagon cells in the human gastrointestinal tract have been published. Evidence of the presence of glucagon-like bioactivity and immunoreactivity in the human colon was provided by *Markman & Sutherland* (1964) and *Samols et al.* (1966)

Accordingly we examined human colon biopsies for glucagon cells of pancreatic type and gut type

Material and Method

Biopsies 1 Ten colon biopsies obtained 15–20 cm from the anus five young and middle-aged persons who on the suspicion of gastrointestinal diseases underwent routine rectoscopy Radiological examination showed normal conditions and histologically the biopsies were found to be without any changes. 2. Sixteen pancreatic specimens obtained 12 hours post mortem from eight middle-aged patients with normal pancreas. For comparison,

fresh pancreatic samples were obtained from normal, six-week-old C-balb mice. All specimens were immediately fixed in Bouin's fluid for 12 hours, embedded in paraffin and cut 1 to 5 μ thick sections.

Antisera. Antisera against porcine glucagon covalently coupled to bovine serum albumin were raised in rabbits (*Holst & Aasted* 1974). The specificity of antiserum no. 4316 to pancreatic glucagon and the complete cross reactivity of antiserum no. 4304 with gut and pancreatic glucagon has been demonstrated previously (4, 5).

Immunohistochemistry. The sections were rinsed in several changes of phosphate buffered saline (PBS) and subjected to the indirect immunofluorescence staining and indirect immunoperoxidase staining according to *Avrameas* (1969). The first layer consisted of either antiglucagonserum no 4303 or no. 4316. The antisera were diluted 20 times and 10 times, respectively with PBS. After rinsing in several changes of PBS a second layer of swine antirabbit globulin labelled with FITC respectively peroxidase diluted 15 times (Dakopath A/S Copenhagen) was applied for 30 minutes and the sections were rinsed in PBS. Subsequently the peroxidase sections were exposed in a benzidin solution for 20 minutes and, after treatment with osmium tetraoxid, the sections were dehydrated in alcohol and mounted.

Controls were treated identically except for that a) the first layer was omitted, b) the second layer was omitted, c) the first layer was replaced by normal rabbit non-immune serum, d) the antiglucagon serum was left to react with glucagon (Glucagon, NOVO 20 μg/ml) before the first layer was applied. The sections were examined under Leitz Orthoplan microscope. For immunofluorescence a Ploem illumination system (light source 200 Hg lamp) was used. Standard filter no. 3 (peak excitation at 490 nm) was used. The immunoperoxidase sections were examined under the microscope in ordinary light.

Result

The background fluorescence and the background discolouration in peroxidase stained sections were minimal. All control sections were negative. The glucagon-immunoreactive cells were demonstrable in all samples, [illegible] specified below no samples were negative.

Pancreas. The pancreatic [illegible]ucagon cells in the islets of Langerhans were equally distinct whether demonstrated by one or the other immunohistochemical technique and by antisera in the human or the mouse pancreas (Fig. 1).

Colon. By both techniques and antiserum no. 4304 cells showing glucagon-immunoreactivity were demonstrable in the human colonic mucosa (Figs. 2–4). Counting included 300 crypts in 10 biopsies. On an average 3.3 cells (range 1–5) were found per crypt mostly in the middle and deeper parts. The cells were conical or triangula[r], the base was resting on the basement membrane and the long axis ran perpendicular to this membrane (Fig. 3). The cytoplasma was finely granular, the immunoreactivity being strongest at the base. The nucleus was round to slightly oval [a]nd located in the middle or in the luminal part of the cell.

If antiserum no. 4316 was used on serial sections to the above-mentioned, no more than, on an average, 1.5 cells (range 1–3) per crypt would constantly be identified when counted in the same way. These cells had exactly the same appearance as those previously described. Consequently on an average twice as many cells reacted with antiserum no. 4304 as with antiserum no. 4316.

Conclusion

Cells showing glucagon-immunoreactivity of pancreatic type and cells showing glucagon-immunoreactivity of g[u]t type have thus been identified in the mucosa of the human colon.

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THE BASEMENT MEMBRANE OF THE UTERINE CERVIX IN DYSPLASIA AND SQUAMOUS CARCINOMA AN IMMUNOFLUORESCENT STUDY WITH ANTIBODIES TO BASEMENT MEMBRANE ANTIGEN

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Rubio C. A. & Biberfeld, P The *basement membrane of the uterine cervix in dysplasia and squamous carcinoma an immunofluorescent study with antibodies to basement membrane antigen*. Acta path. microbiol scand. Sect. A, 83 744–748, 1975

Histological preparations with normal epithelium, dysplasia, carcinoma in situ and squamous invasive carcinoma of human cervix were studied by immunofluorescence using human sera containing antibodies against basement membrane material against intercellular substance of squamous epithelium (ISSE) and against reticulin. A continuous basement membrane was observed in normal epithelium, dysplasia and carcinoma in situ, but was faintly stained, discontinuous or even absent in specimens with invasive squamous carcinoma. Staining with anti-reticulin did not reveal any condensation in the basement membrane areas *in any of the* preparations examined. ISSE was demonstrated in the normal epithelium, dysplasia and carcinoma in situ as well as in invasive carcinoma.

Key words: Immunofluorescence basement membrane cervix cancer

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According to several authors the basement membrane of the uterine cervix as seen by light microscopy is composed of condensed reticulin or/and periodic acid-Schiff (PAS) positive ground substance at the junction of the stroma with the epithelial coat (*Schiller et al.* 1953 *Younes et al* 1965 *Marcuse* 1971) Both reticulin and PAS positive ground substance have been shown, however to be discontinuous or even absent in cases with (*Hampert* 1967) or without (*Lamb et al.* 1960) inflammation of the cervical tissue. These findings led *Lamb et al.* to conclude that "no true basement membrane is present under the stratified squamous epithelium and columnar epithelium of the cervix (*Lamb et al.* 1960)

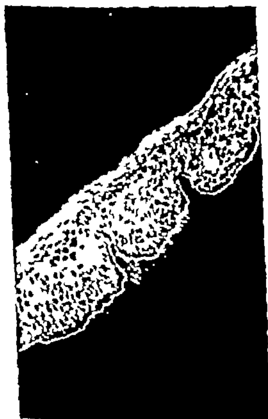
By using an immunofluorescent technique, *Beutner et al* (1968) demonstrated, however the presence of a thin continuous basement membrane-like zone beneath various normal squamous epithelia such as skin, oesophagus and vagina, which reacted with antibodies present in the sera from patients with bullous pemphigoid. In the present study these investigations have been extended to the uterine cervix

Five micrometer thick tissue sections of 3 cone specimens and of 3 punch biopsies from cervical tumours were studied with the indirect fluorescent technique using sera from patients with bullous pemphigoid having high titres of antibodies against epithelial basement membrane (EBM) The histological diagnosis made on H & E frozen sections in the cone specimens was dysplasia or carcinoma in situ and in the punch biopsies invasive squamous carcinoma

The results demonstrated in all sections from cone specimens the presence of a regular continuous, sharply demarcated, fluorescent linear struc



Fig 1 Immunofluorescent basement membrane beneath the cervical epithelium. (250×)



ture beneath the normal cervical epithelium (squamous and columnar) (Fig 1) as well as beneath epithelium with dysplasia and carcinoma in situ (Fig. 2) The continuity of the membrane was confirmed on multiple sections several mm apart from each other and in sections cut at various angles in the same specimen. The study of the 3 cases of invasive squamous carcinoma of the cervix demonstrated in one case with moderate differentiated squamous carcinoma (Fig 3) the presence of a fragmented, faintly fluorescent basement membrane surrounding clusters of invasive cells. In the two remaining cases with poorly differentiated squamous carcinoma no fluorescent basement membrane surrounding invasive clusters could be demonstrated (Fig 4) Similar results have been obtained in two cases with invasive squamous carcinoma of the lung (*data to be published*)

In order to investigate the relationship between epithelial components and the basement membrane sections of cone specimens were examined by indirect immunofluorescence after treatment with antibodies directed against intercellular substance of squamous epithelium (ISSE) present in sera from patients with pemphigus foliaceus (*Ali & Wright 1971*) Normal cervical epithelium, dys-

Fig. 2 Immunofluorescent basement membrane beneath dysplastic epithelium. The specimen was also treated with antibodies against ISSE. (100×)

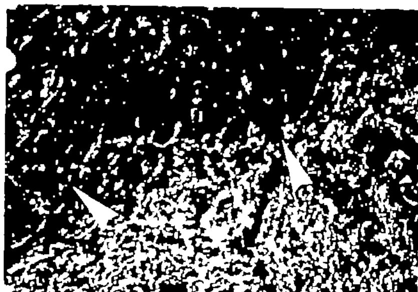


Fig 3 Cluster of invading cells in a case with moderate differentiated squamous carcinoma. Note discontinuity of the immunofluorescent basement membrane at arrows. (250 ×)



Fig 4 Two clusters of invading cells in a case with poorly differentiated squamous carcinoma. Note absence of immunofluorescent basement membrane at arrows. (250 ×)

plasia and carcinoma in situ demonstrated a sharply delineated, fluorescent network surrounding normal (Fig 5) and atypical squamous cells (Fig. 2). In no case a continuous fluorescent line beneath the epithelium was observed. Consecutive sections in the same cases demonstrated that anti-basement membrane fluorescent antibody reacted specifically with the basement membrane confirming previous observations that EBM and ISSE are different antigens, structurally independent. The intercellular substance was clearly demonstrated also in areas with invasive carcinoma, even in cases where the basement membrane was totally absent (Fig 6).

The relationship between the basement membrane and the stromal reticulin was studied by indirect immunofluorescence using antireticulin



Fig 5 Immunofluorescent intercellular substance in the normal squamous epithelium of the cervix. (250×)

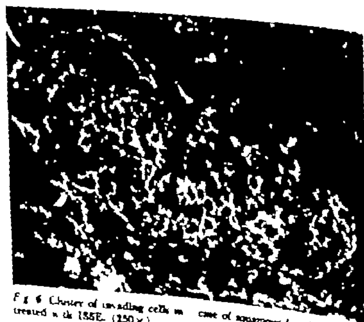


Fig 6 Cluster of invading cells in case of squamous invasive carcinoma treated with ISSE. (250×)

were obtained from patients with malabsorption syndrome (Ip & Wright 1971). In these experiments fluorescent threads were observed in the stroma beneath the normal epithelium, dysplasia and carcinoma in situ and sometimes surrounding the epithelial cords. In no case continuous fluorescent line as that seen with anti-ERM was found.

The above mentioned studies have demonstrated the presence of a continuous basement membrane as defined by anti-ERM antibodies, beneath the normal epithelium of the cervix and

dysplasia and carcinoma in situ. In the cases so far investigated with invasive squamous carcinoma that structure was discontinuous, faintly fluorescent or even absent in invasive areas possibly depending on the degree of tumour differentiation. Similar observations as those in human material have been made in the cervix of mice, both in the normal squamous epithelium, in epithelium with various degree of atypia as well as in invasive squamous carcinoma (data to be published)

The observations with anti-R-antibodies showed no condensation of reticulin in the basement membrane. This is in contradiction with the findings of other authors after conventional histochemical staining procedures. The possibility that our anti-R-serum is restricted in its specificity has been considered and several other sera will be tested.

Another aim of future work is to investigate the characteristics of the immunofluorescent basement membrane in areas with microinvasive carcinoma of the cervix both in mice (*Rubio & Lagerlöf* 1974) and in human subjects (*Rubio et al.* 1974)

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